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Cerebral edema and acute liver failure: pathophysiological mechanisms and new therapeutic approaches

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Cerebral edema and acute liver failure: pathophysiological mechanisms and new therapeutic approaches

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ABSTRACT

Hepatic encephalopathy (HE) contains a spectrum of neuropsychiatric abnormalities observed in patients with liver disease. A quick worsening of consciousness and increasingly growing cerebral edema, high intracranial pressure, which leads to cerebral herniation and death, are characteristics of acute liver failure (ALF). Multiple factors are found responsible for the development of HE, whereas, over 100 years, hyperammonia is considered the most crucial factor in defining the pathogenesis of HE in ALF, which can increase to millimolar concentrations in the brain at the coma stages of HE.

The present thesis comprises 4 articles, which demonstrates new pathogenic mechanisms involved in the development of HE and cerebral edema in ALF, and elucidates part of the therapeutic mechanism of hypothermia and minocycline in the prevention of HE and cerebral edema during ALF. The major findings are listed below:

(1) Experimental ALF leads to the increase in brain production of proinflammatory cytokines (IL-6, IL-1β, TNF-α), and provides the first direct evidence that central inflammatory mechanisms play a role in the pathogenesis of the encephalopathy and brain edema in ALF (chapter 2.1 - article 1; chapter 2.1 - article 2).

(2) Activation of cerebral microglia, measured by OX-42, OX-6, predicts the presence of severe encephalopathy (coma) and brain edema in rats with ischemic ALF, which

accompanies the increased production of brain proinflammatory cytokines (chapter 2.1 - article 1; chapter 2.2 - article 2).

(3) Oxidative/nitrosative stress participates in the pathogenesis of brain edema and its complications in experimental ALF animals with ischemic liver failure. The increases in cerebral NOS isoform expression caused by ALF were sufficient to cause increased NO production in the brain (chapter 2.3 - article 3; chapter 2.4 - article 4).

(4) Anti-inflammatory treatment, such as hypothermia or antibiotics, may be beneficial in patients with ALF (chapter 2.1 - article 1; chapter 2.2 - article 2).

(5) The beneficial effect of both hypothermia and minocycline on the neurological complications of experimental ALF is mediated, at least in part, by reduction of brain-derived oxidative/nitrosative stress (chapter 2.3 - article 3; chapter 2.4 - article 4).

Key words: Acute liver failure, Hepatic encephalopathy

RÉSUMÉ

L'encéphalopathie hépatique (EH) se développe chez les patients atteints d'une maladie du foie et se caractérise par de nombreuses anomalies neuropsychiatriques. L'insuffisance hépatique aiguë (IHA) se caractérise par une perte progressive de l'état de conscience, par une augmentation rapide de l'œdème cérébral et une augmentation de la pression intracrânienne entraînant une herniation cérébrale et la mort. Plusieurs facteurs sont responsables du développement de l'EH mais depuis une centaine d'années, l'hyperammonémie qui peut atteindre des concentrations de l'ordre de plusieurs millimolaires chez les patients atteints d'IHA aux stades de coma est considérée comme un facteur crucial dans la pathogenèse de l'EH.

La présente thèse comprend 4 articles suggérant l'implication de nouveaux mécanismes pathogéniques dans le développement de l'EH et de l'œdème cérébral associés à l'IHA et tente d'expliquer l'effet thérapeutique de l'hypothermie et de la minocycline dans la prévention de l'EH et de l'œdème cérébral:

1. L'IHA induite par dévascularisation hépatique chez le rat se caractérise par une augmentation de la production de cytokines pro-inflammatoires cérébrales (IL-6, IL-1β, TNF-α). Cette observation constitue la première évidence directe que des mécanismes neuro-inflammatoires jouent une rôle dans la pathogenèse de l'EH et de l'œdème cérébral associés à l'IHA (Chapitre 2.1, articles 1 et 2).

2. L'activation de la microglie telle que mesurée par l'expression de marqueurs spécifiques (OX42, OX-6) coïncide avec le développement de l'encéphalopathie (stade coma) et de l'œdème cérébral et s'accompagne d'une production accrue de cytokines pro-inflammatoires cérébrales (Chapitre 2.1, article 1 et 2).

3. Un stress oxydatif/nitrosatif causé par une augmentation de l'expression de l'oxyde nitrique synthétase et une augmentation de la synthèse d'oxyde nitrique cérébral participe à la pathogénèse des complications neurologiques de l'IHA (Chapitre 2.3, articles 3 et 4).

4. Des traitements anti-inflammatoires tels que l'hypothermie et la minocycline peuvent constituer de nouvelles approches thérapeutiques chez les patients atteints d'IHA (Chapitre 2.1, article 1; Chapitre 2.2, article 2).

5. Les effets bénéfiques de l'hypothermie et de la minocycline sur les complications neurologiques de l'IHA expérimentale s'expliquent, en partie, par une diminution du stress oxydatif/nitrosatif (Chapitre 2.3, article 3; Chapitre 2.4, article 4).

Mots Clefs: Insuffisance hépatique aiguë, encéphalopathie hépatique

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LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
α-KG	alpha-ketoglutarate
α-KGDH	alpha-ketoglutarate dehydrogenase
AAA	Aromatic amino acids
AAT	Aspartate aminotransferase
AD	Alzheimer`s disease
ALA	Alanine
ALF	Acute liver failure
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMP	Adenosine monophosphate
ANA	Antinuclear antibodies
ANC	Adenine nucleotide carrier
APAP	acetaminophen
ASP	Aspartate
ATP	Adenosine triphosphate
BAL	Bioartificial liver device
BBB	Blood-brain barrier
BCAA	Branched-chain amino acids
BPIA	Benzodiazepine partial inverse agonists
BZD	Benzodiazepine

Ca^{2+}	Calcium
CBF	Cerebral blood flow
CCl_4	Carbon tetrachloride
cGMP	Cyclic guanosine monophosphate
CH	Cerebral herniation
Cl^-	Chloride
CLF	Chronic liver failure
CMR_A	Cerebral metabolic rate for ammonia
$CMR_{glucose}$	Cerebral metabolic rate for glucose
CNS	Central nervous system
CO	Carbon monoxide
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CPP	Cerebral perfusion pressure
CsA	Cyclosporin A
CSF	Cerebrospinal fluid
CVVHD	Continuous venovenous hemodiafiltration
DA	Dopamine
DBI	Diazepine binding inhibitor
DON	6-diazo-5-oxo-L-norleucine
DSH	Deliberate self-harm
EAAT-1	Excitatory amino acid transporter 1
EAAT-2	Excitatory amino acid transporter 2

EAE	Experimental allergic encephalomyelitis
EEG	Electroencephalogram
eNOS	endothelial nitric oxide synthase
FHF	Fulminant hepatic failure
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCMS	Gas chromatography-mass spectrometry
GCS	Glasgow coma scale
GDH	Glutamate dehydrogenase
GFAP	Glial fibrillary acidic protein
GLU	Glutamate
GLT1	Glutamate transporter 1
GLN	Glutamine
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
GLY	Glycine
GLYT-1	Glycine transporter 1
GS	Glutamine synthetase
GSH	Glutathione/gamma-L-Glutamyl-L-cysteinyl-glycine
H^+	Hydrogen
HA	Hyperammonemia
HAL	Hepatic artery ligation
HAV	Hepatitis A virus

HBV	Hepatitis B virus
HCV	Hepatitis C virus
HE	Hepatic encephalopathy
HEV	Hepatitis E virus
HO-1	Heme oxygenase-1
HVA	Homovanillic acid
IBP	isoquinoline binding protein
ICH	Intracranial hypertension
ICP	Intracranial pressure
IFN-γ	Interferon gamma
IL-1β	Interleukin-1β
IL-6	Interleukin-6
ILE	Isoleucine
iNOS	Inducible nitric oxide synthase
INR	International normalized ratio
IPSP	Inhibitory postsynaptic potential
K^+	Potassium
KA	Kainate
Lac	Lactate
L-DOPA	Levodopa
LEU	Leucine
L-NNA	N-omega-nitro-L-arginine
LOLA	L-ornithine-L-aspartate

MAO-A	Monoamine oxidases-A
MAP	Mean arterial pressure
MAP-2	Microtubule-associated protein 2
MARS	Molecular adsorbent recirculating system
MCP-1	Monocyte chemotactic protein-1
mHE	minimal hepatic encephalopathy
ME	Malic enzyme
MELD	Model for end-stage liver disease
MIP-1	Macrophage inflammatory protein-1
Mn^{2+}	Manganese
MPT	Mitochondrial permeability transition
MR	Magnetic resonance
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSO	Methionine sulfoximine
NAC	N-Acetylcysteine
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NAFLD	Nonalcoholic fatty liver disease
NH_3/NH_4^+	Ammonia
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
NO	Nitric oxide

NOS	Nitric oxide synthase
NOx	nitrite/nitrate
nNOS	Neuronal nitric oxide synthase
OA	L-ornithine-L-aspartate
OAT	Ornithine aminotransferase
ODN	Octadecaneuropeptide
OLT	Orthotopic liver transplantation
PAG	Phosphate-activated glutaminase
PC	Pyruvate carboxylase
PCA	Portacaval anastomosis
PCS	Portacaval shunted
PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PHE	Phenylalanine
PMA	Phorbol-12-myristate-13-acetate
POST	Postsynaptic neuron
PRE	Presynaptic neuron
PS	Permeability surface
PSE	Portal-systemic encephalopathy
PTBR	Peripheral-type benzodiazepine receptor
QRT-PCR	quantitative real-time reverse-transcription polymerase chain reaction
QUIN	Quinolinic acid
RNOS	Reactive nitrogen oxygen species

ROS	Reactive oxygen species
SBP	Spontaneous bacterial peritonitis
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
sPLA2	Phospholipase A2
SVR	Systemic vascular resistance
TAA	Thioacetamide
Tau	Taurine
TCA cycle	Tricarboxylic acid cycle
THDOC	3α-5α-tetradehydrodeoxy-corticosterone
TIPSS	Transjugular intrahepatic portal-systemic shunt
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TRIM	1-2-trifluoromethylphenyl imidazole
TRP	Tryptophan
TSPO	Translocator protein
TYR	Tyrosine
VAL	Valine

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CHAPTER 1

REVIEW OF THE LITERATURE

1. Introduction

HE is defined as: “a spectrum of neuropsychiatric abnormalities seen in patients with liver dysfunction after exclusion of other known brain diseases”, a consensus reached in 1998 [1].

1.1 Classification and grading of HE

1.1.1 Type A, B and C

Three types of HE should be recognized on principle:

Type A - encephalopathy associated with ALF

Type B - encephalopathy associated with portal-systemic bypass and no intrinsic hepatocellular disease

Type C - encephalopathy associated with cirrhosis and portal hypertension

Type A and type C HE are seen in patients with acute and chronic liver failure, respectively. Type C HE is also referred to as portal systemic encephalopathy (PSE). Unlike type A and C, type B HE is a very rare disease, resulting from the portal-systemic bypass rather than a disease primarily involving the liver itself. In more complex clinical cases, especially those involving type B and C, HE can be further

classified into groups of episodic (recurrent), persistent, and minimal based on duration and characteristics of the clinical symptoms:

(1) In episodic (recurrent) HE, neurological symptoms are seen in an episodic and recurrent fashion in cirrhotic patients, which could be independent of the presence of precipitating factor(s).

(2) In persistent HE, neurological symptoms persist even after the removal of precipitants. It also includes treatment-dependent HE, in which discontinuation of the medication leads to persistent neurologic symptoms.

(3) Minimal encephalopathy, previously named “subclinical encephalopathy” is found in approximately 70% of patients suffering from liver disease and characterized by mild cognitive impairment [2]. Although minimal encephalopathy can be detected only by using specialized neurological tests, this type of HE increases the chances of patients getting involved in traffic accidents [3].

Regarding ALF, it is further classified, according to the interval between the onset of symptoms (such as jaundice) and the development of encephalopathy [4].

Table 1: Classification of fulminant liver failure
Fulminant liver failure (formation of ascites or HE within weeks after onset of symptoms)
a) hyperacute: 0 to 7 days
b) acute: 8 to 28 days
c) subacute: 5 to 24 weeks

1.1.2 Clinical staging

The West Haven Criteria is the most widely used criteria for grading HE [1, 5]. Although originally developed for clinical classification of cirrhotic patients, it can also be used to classify ALF patients. In both cirrhotic patients [6] and ALF patients [7], the grading of HE could be useful information in predicting the mortality rate and deciding the need for liver transplantation.

Table 2: West Haven Criteria for grading mental state in HE [5]

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Grade 0 - No psychometric and/or neurophysiological abnormality can be detected.

Grade 1 - trivial lack of awareness; shortened attention span; euphoria or anxiety; irritability; psychomotor slowing, impaired performance of addition and subtraction; reversal of sleep rhythm; slight disturbances of mood and personality, untidiness

Grade 2 - drowsiness, lethargy, apathy; intermittent disorientation, disorientation for

time or place; major mental disabilities; obvious personality change; inappropriate behavior

Grade 3 - somnolence to semi-stupor, but responsive to stimuli; pronounced confusion; persistent disorientation, gross disorientation, incoherent speech with perseveration, unable to perform mental tasks

Grade 4 - coma, with (IVa) or without (IVb) response to painful stimuli, unresponsive to verbal stimuli, mental state not testable.

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Glasgow coma scale (GCS) was initially designed for neurotrauma patients [8, 9], which provides better grading than the West Haven Criteria when HE patients reach its grade 3 or 4 [10]. Patients are evaluated for eye, verbal and motor capabilities in the GCS. It ranges from 3 (the worst, deep coma) to 15 (the best, awake person) points. GCS score of equal or over 13 is considered as minor cerebral injury. GCS score of 9 to 12 indicates a moderate cerebral injury. GCS score of equal to or less than 8 suggests severe cerebral injury (= coma).

1.2 Epidemiology and etiology of ALF

ALF is a rare disease. Only approximately 2000 ALF cases per year are reported in the US [11]. The causes of ALF play a significant role in the survival rate and the

need for liver transplant, in which the survival rate is 25% in idiosyncratic drug reaction and 68% in acetaminophen overdose, whereas the percentage of ALF patients receiving liver transplantation was 53% in idiosyncratic drug reaction, and 6% in acetaminophen overdose.

10% of all liver transplantations in the US are performed to treat ALF, in the Far East, approximately 66% of all transplantations are performed to treat ALF and acute-on-chronic liver failure. The most common causes of ALF are drug-intoxications in the western world, however, in the Far East, ALF due to chronic HBV is the most common cause, such as in Hong Kong, which accounts for 79% of cases, whereas ALF due to hepatitis E and hepatitis B infection accounts for 38% and 31% respectively in India [12]. Chronic Hepatitis B infection may flare up following a super-infection by other viruses, or after cytotoxic or immunosuppressant therapy [13]. Although HCV infection is a common (>50%) cause of hepatitis virus infection in China [14], it is generally not considered to result in ALF alone. In total, only 0.2%-4% of all patients infected with hepatitis viruses eventually develope ALF [11, 14].

2. Neuropathology of HE type A

As early as the 1970s, Ware [15] and Williams [16] reported that brain edema is a unique feature of ALF, and recognized that brain herniation is the cause of death in ALF patients after studying autopsied brain samples from ALF patients. Later studies revealed

that the classic neuropathologic feature of ALF is cytotoxic brain edema, demonstrated by the swelling of astrocytes and their processes [17, 18]. Likewise, astrocyte swelling has been consistently reported in various animal models of ALF at coma stages [18-23]. Astrocyte swelling is more obvious in foot processes than in cell bodies [18, 19]. Swollen astrocytes and brain edema are more prominent in cortical gray matter than in subcortical white matter [18, 19]. In contrast to the change of astrocytes, no neuronal morphologic alteration was found in the brain of ALF [20, 21].

Astrocyte swelling and cerebral edema could be severe enough to increase intracranial pressure (ICP), which leads to brain herniation - the major cause of death in ALF [24, 25]. The incidence of brain herniation is positively correlated with elevated arterial ammonia levels in ALF patients [26]. In recent studies, evidence of cerebral edema is provided by diffusion-weighted MRI [27] and CT scanning [28] methods in ALF patients with deep encephalopathy.

3. Pathogenesis of HE type A

Initial attempts to investigate the pathogenesis of HE with ALF assumed that the neurologic impairment was induced by blood-borne toxins, not cleared by the failing liver. From the 1960s to the 1980s, theories of synergistic toxins [29] (ammonia, mercaptans, phenols, short-chain fatty acids, octanoic acid), GABA alterations and energy failure have been proposed and extensively studied. Brain edema was viewed

as a distinct feature of ALF from the 1970s [30], which has been since treated as an important pathophysiologic end point in experimental studies [31]. Although the major causative factors (such as: ammonia) leading to HE have been recognized, their precise mechanisms are incomplete or elusive and other new factors are continually being discovered.

3.1 Ammonia: a gut-derived toxin

3.1.1 The crucial role of ammonia in the pathogenesis of HE

Ammonia is the most critical factor in the pathogenesis of HE. Ammonia levels increase in the cerebrospinal fluid (CSF) and the blood in HE patients [32]. In one study, the influx of ammonia into the brain was 45-fold higher in experimental ALF animals than those of controls [33]. Further evidence that supports the role of ammonia in the development of HE includes: (1) Administration of ammoniagenic substances to cirrhotic patients quickly induces HE symptoms [34]. (2) Hyperammonemia induces HE-like symptoms in subjects with healthy livers (such as: in urea-cycle defect [35, 36]). (3) Blood ammonia levels correlate well with the occurrence of cerebral edema, increased ICP and cerebral herniation in ALF [26, 37, 38]. (4) Treatments which reduce blood ammonia, such as lactulose, antibiotics, L-ornithine-L-aspartate (LOLA), hypothermia, improve HE [5, 32, 39, 40].

3.1.2 Biochemical features of ammonia

Ammonia, depending on pH, can be present as an ion (NH_4^+) that cannot pass the membranes unless bound to carriers [41, 42], or appears as lipophilic gas (NH_3) which can freely travel across membranes. Ammonia is involved in many enzymatic reactions, but could also be produced as a by-product of other reactions.

Ammonia (NH_3) remains in equilibrium with the ammonium ion (NH_4^+) in aqueous solutions ($NH_3 + H^+ \leftrightarrow NH_4^+$) [41, 42]. The ratio of NH_3 to NH_4^+ is influenced by pH as described by the Henderson-Hasselbach equation ($Log_{10}[\frac{NH_3}{[NH_4^+]}] = pH - pK_a$). Thus, under normal physiological conditions of pH 7.4, over 98% of ammonia is present as NH_4^+, because the pKa of ammonia is 9.15 at 37^{o}C [43]. "Ammonia" in this thesis refers to total ammonia ($NH_3 + NH_4^+$) levels.

Although a large amount of ammonia produced after the digestion of a protein diet enters the portal vein, the liver removes most of the gut-derived ammonia and maintains arterial ammonia concentration at a low level (~50μM) [44-46]. Liver dysfunction results in increased circulating ammonia levels in HE. Inter-organ trafficking of ammonia in normal physiological condition versus ALF is shown in Figure 1.

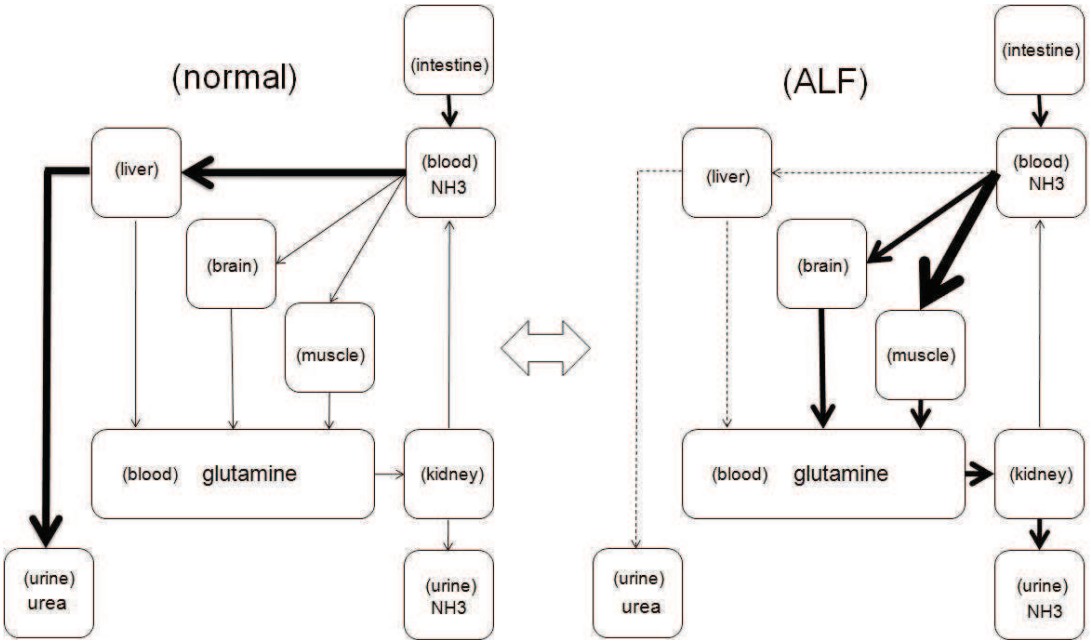

Figure 1: Interorgan trafficking of ammonia during normal conditions and ALF. In normal conditions, ammonia produced by the gut is removed by the liver as urea or glutamine. Urea is excreted into urine. Hence, arterial ammonia concentration is tightly controlled. In ALF, liver substantially decreases the capacity to detoxify ammonia. The elevated arterial ammonia leads to increased ammonia in muscle and brain, which lack a complete urea cycle and rely on glutamine synthesis. Glutamine is thus increasingly released from muscle and brain into blood in ALF. Quantitatively, muscle becomes the major ammonia detoxifying organ in ALF. Kidney shifts from mainly ammonia-releasing into blood (in normal condition) to mostly ammonia excretion into urine in ALF, but this way of ammonia excretion by kidney is insufficient to remove total blood ammonia. With consistent ammonia production from gut and limited capacity for muscle to extract ammonia, blood ammonia levels rise in ALF.

3.1.3 Blood ammonia concentrations

The arterial ammonia level in normal individuals is approximately 50μM [44]. It increases to approximately 165μM in ALF patients [26, 44] and rises to 400μM in ALF patients in deep coma with uncontrolled high ICP [26, 39]. In ALF animals, blood ammonia may increase to 1mM, which is 15-20 times higher than those of controls [47, 48].

As an ammonia detoxification product, blood glutamine can increase from 600μM in healthy individuals to 2500μM in ALF patients [44]. In ALF animals, blood glutamine levels could increase 5 times as high as those of the control [33].

3.1.4 Brain ammonia uptake and concentrations

Ammonia free base (NH_3) moves across cell membranes more easily than NH_4^+. As the NH_4^+ to NH_3 conversion rate ($4.3\times10^{10}\ M^{-1}\ s^{-1}$) is so rapid [42], it has been calculated that 75% of ammonia enters the brain as NH_3 and 25% as NH_4^+. There is a strong correlation ($r = 0.80$) between arterial ammonia concentration and cerebral ammonia extraction (arteriovenous difference) in ALF patients [26, 49]. However, it has been observed that the rate of ammonia uptake by the brain does not correlate well with arterial ammonia concentration over a wide range [50], which indicates the involvement of other factors rather than blood ammonia concentrations alone.

Based on large clinical and experimental observations, a theory of pH-dependent cerebral ammonia uptake has been proposed. According to the Henderson-Hasselbach equation, assuming that the pH of blood is 7.4 and cerebral pH is 7.1 [42], the normal ratio of brain to blood ammonia is 2, which is consistent with that found in normal animals of a ratio 1.5 to 3 (~0.1mM NH_3 in the brain). In ischemic ALF, CSF pH continually decreases when HE worsens, and can reach to as low as 6.8 as the animal enters coma stage. The decrease in CSF pH is considered due to an increase in cerebral lactate concentration (see "energy metabolism" section for details). Accordingly, the brain to blood ammonia ratio increases in ALF, and could rise to as high as 8 in hyperammonemic conditions [51]. In practice, cerebral ammonia levels of 1-5mM have been described in various ALF animal models at their comatose stage [31, 52-55]. A similar increase of cerebral ammonia concentration has been observed in ALF patients [26].

3.1.5 Brain ammonia metabolism

Due to the lack of carbamoyl-phosphate synthase I and ornithine transcarbamylase, brain cannot remove ammonia with the formation of urea. The predominant way for brain ammonia removal in both normal and hyperammonemic conditions is by the conversion of ammonia and glutamate into glutamine via glutamine synthetase (GS) [56]. Glutamine then can be either stored in the brain or secreted into the blood [42, 57-59]. In ALF rats, 66% of cerebral extracted ammonia is metabolized, probably into glutamine, 33% of this is stored in cerebral ammonia pools [33]. Consequently, cerebral

glutamine concentrations are elevated 2 to 5 times in HE animals as well as in HE patients [60-66]. Evidence of the exclusive localization of GS in astrocytes [67, 68] indicates that astrocytes are important cells responsible for ammonia detoxification.

On the other hand, several mechanisms have been proposed to explain the incomplete cerebral ammonia removal in brain in ALF: (1) Unlike skeletal muscle, GS expression in the brain is not significantly altered in hyperammonic conditions [69, 70], (2) Cerebral GS activity is fully functioning during normal physiological situations [42], and is not significantly increased further in hyperammonia states.

3.1.6 Ammonia metabolism in muscle during liver failure

Increased muscle ammonia uptake has been reported in ALF patients [71]. A net ammonia consumption by muscle (100 nmo1/100g/min) has been reported in ALF patients [44], which correlates with muscle glutamine production. So far, ammonia uptake by the muscle has been mostly reported in subjects with decompensated ALF. Muscle wasting decreased the rate of ammonia extraction in liver failure patients [71, 72], which indicates an important role of muscle in ammonia-detoxification.

Whether muscle glutamine release is stoichiometrically related to muscle ammonia uptake is a controversial issue. Using various animals, increased muscle ammonia uptake has been observed in ammonia-infused animals [73], concomitant with either increased muscle glutamine production [74] or no alteration [75]. In a human study,

muscle glutamine production was 10 times greater in proportion to the amount of muscle ammonia uptake, which indicates a catabolic state in ALF [44].

In conclusion, muscle plays an important role in ammonia removal in decompensated ALF due to the muscle's large organ mass.

3.1.7 Hyperammonia-induced brain edema and astrocyte swelling, in type A HE

Increased arterial ammonia levels in ALF patients are associated with higher incidence of brain herniation and increased ICP [26]. The close association between high cerebral ammonia (1-5mM) and the development of cerebral edema was also observed in ALF animals [31]. Treatment of cerebral cortical slices with relevant levels of ammonia seen in comatose animals results in swelling and reduction of inulin space [76]. Significant astrocyte swelling was also demonstrated in ammonia-administrated primates [77] and ammonia-treated cultured astrocytes [78].

3.1.8 Glutamine hypothesis in association with hyperammonia-induced cerebral edema and HE

It is generally accepted that ammonia is the most important causative factor leading to HE [79]. The level of hyperammonia has been found strongly associated with cerebral function [66, 80], clinical stages [81]. However, a direct cause-effect relationship

between ammonia and HE has been questioned by a few researchers [81-84]. A lack of good correlation between cerebral ammonia and HE grade has been described in clinical studies [42]. In addition, administration of a GS inhibitor to ALF animals prevents the development of cerebral edema and high ICP, but with an unexplained further increase in blood and cerebral ammonia [85-87]. Therefore, hyperammonia has been viewed as necessary and sufficient to induce cerebral edema in ALF, but the harmful effects of hyperammonia were attributed to ammonia's cerebral metabolites rather than by ammonia itself [88]. As an ammonia metabolite, glutamine is proposed as a candidate responsible for the development of cerebral edema and HE in ALF.

The evidence of a harmful role of glutamine in ALF is largely provided by studies using the GS inhibitor: methionine sulfoximine (MSO), which decreases cerebral glutamine levels [89]. Giving MSO to hyperammonia-exposed animals reduced their death rate [89], ameliorates cerebral edema [85, 86], high ICP [85], astrocyte swelling [90-92] and high CBF [93], restores cerebral vascular autoregulation [94, 95] and brain glucose metabolism [88, 96, 97], attenuates reactive oxygen species (ROS) production [98, 99] and high mitochondrial permeability transition (MPT) [100, 101]. In addition, cerebral glutamine levels correlate better than ammonia with clinical HE grade and cerebral edema in ALF patients [102, 103] and ALF animals [85, 104, 105].

3.1.8.1 Two glutamine-based hypotheses to explain astrocyte swelling in HE

3.1.8.1.1 Glutamine-osmolyte-edema hypothesis

As the brain lacks a complete urea-cycle, cerebral ammonia detoxification mainly depends on glutamine formation [42, 70]. The responsible enzyme GS is exclusively localized in astrocytes [67]. Glutamine is an osmolyte [106] and its accumulation in astrocytes moves water from the extracellular space into intracellular space [107-109], which could lead to astrocyte swelling and cytotoxic brain edema in ALF.

3.1.8.1.2 Evidence in favour of the ‘‘glutamine-osmolyte-edema hypothesis’’

In support of ‘‘glutamine-osmolyte-edema hypothesis’’, a several-fold increase of cerebral glutamine has been observed in ALF patients [110, 111]. In animal studies, the association between cerebral glutamine accumulation and cerebral edema has been demonstrated in hepatectomized ALF animals [112], ischemic ALF animals [31, 47, 60, 113-115], toxin-induced ALF [105], and hyperammonia-induced HE [57, 58, 85, 116-118]. Using NMR, a 10 fold increase of ^{13}C-labelled glutamine synthesis from glucose in the brain, via the anaplerotic pathway, was observed in ischemic ALF animals [114, 115]. Furthermore, MSO treatment reduced cerebral glutamine and, at the same time, attenuated cerebral edema, high ICP and CBF in ALF animals [85, 86, 93, 96, 119, 120].

3.1.8.1.3 Evidence against the ‘‘glutamine-osmolyte-edema hypothesis’’

Evidence weakening the ‘‘glutamine-osmolyte-edema hypothesis’’ has been presented

in the literature [25, 114, 115, 121-124]. Using advanced [^{1}H-^{13}C] NMR in ischemic ALF animals, the concentration of cerebral glutamine showed no correlation with brain edema [115, 124]. In addition, the improvement of brain edema by hypothermia [114, 121] and indomethacin [24] was not associated with the prevention of cerebral glutamine accumulation.

3.1.8.1.4 Glutamine-RNOS-edema hypothesis

An advanced "glutamine-RNOS-edema hypothesis" was developed recently, in order to explain the imperfect correlation between glutamine and brain edema in HE, and to amend the theory which concerns the role of glutamine in the pathogenesis of HE. In this hypothesis, glutamine is considered as an ammonia carrier. Glutamine moves into astrocytic mitochondria through a histidine-sensitive glutamine transporter [125], which is subsequently hydrolyzed by phosphate-activated glutaminase (PAG) (an inner mitochondrial enzyme) [126], and split into glutamate and ammonia [127, 128]. The production and accumulation of ammonia in the astrocytic mitochondria lead to increased MPT, ROS production, which impairs mitochondrial function, consequently resulting in cell swelling.

3.1.8.1.5 In vitro evidence supporting this "glutamine-RNOS-edema hypothesis"

3.1.8.1.5.1 Glutamine induce MPT and ROS, which subsequently contribute to astrocyte swelling

Addition of glutamine to cultured astrocytes induces the opening of MPT [129] and the production of free radicals [130], which are not observed in cultured neurons. Increased ROS has been shown to lead to cell swelling in cultured astrocytes [131-136] and brain slices [131, 137, 138], and to induce MPT opening, which results in further free radicals generation [139-141]. In the similar experimental setting, the MPT opening-associated astrocyte swelling [100] can be prevented by the treatment with a MPT blocker: cyclosporine [142].

3.1.8.1.5.2 PAG and histidine-sensitive glutamine transporters are required for glutamine-induced MPT, ROS and astrocyte swelling

Administration of 6-diazo-5-oxo-L-norleucine (DON), a PAG inhibitor, to ammonia-exposed cultured astrocytes significantly attenuate ROS generation, MPT opening [130, 143] and astrocyte swelling [144]. In addition, treatment with histidine, a blocker of glutamine-mitochondria-transporter [125], attenuates ROS production, MPT opening and astrocyte swelling in ammonia-exposed cultured astrocytes [145].

3.1.8.1.5.3 Realization and limitation of in vitro findings

The presence of PAG has been demonstrated in both cultured astrocytes [146-148] and animal cerebral samples using molecular bio-techniques [149-151], which indicates the likelihood to transfer in vitro finding to interpret results obtained from animal

experiments. However, currently, almost all evidence in support of "glutamine-RNOS-edema hypothesis" are obtained from in vitro experimental settings [31, 152, 153], which rises caution for the over-interpretation of in vitro findings.

3.2. Brain energy metabolism in ALF

In general, no evidence of loss of ATP has been demonstrated in clinical ALF. However, altered cerebral glucose metabolism has been clearly shown.

3.2.1 Evidences of changed glucose metabolism in ALF

Elevated cerebral and CSF lactate has been consistently described in various ALF animal models [31, 47, 112, 113, 115, 154-156], and in ALF patients [157-159]. H-/C-/P-NMR studies confirm the increased de novo synthesis of cerebral lactate from glucose in ALF animals [114, 115, 155]. The increased cerebral lactate level is positively correlated with the worsening of HE in ALF [47, 154, 156, 160-163]. Moreover, increased cerebral alanine has been demonstrated in hyperammonemic animals using biochemical methods [31, 47, 61, 164] or NMR [114]. Both of the increases in cerebral lactate and alanine indicate impaired cerebral pyruvate oxidation in ALF. (In addition, increased alanine could result from ammonia incorporation into glutamate by alanine aminotransferase.) As expressed, brain high-energy phosphates (phosphocreatine and ATP) were unaltered in ALF animals measured by biochemical approach [47, 112] or ^{1}H-/^{31}P-NMR [47, 112, 154, 155, 165].

3.2.2 Hypothesis for altered cerebral glucose metabolism in ALF

The most popular hypothesis is that hyperammonia inhibits a-ketoglutarate dehydrogenase (aKGDH) which impairs the TCA cycle and compromises energy metabolism, resulting in increased cerebral lactate accumulation in ALF [166] (Figure 2). As early as 1961, the accumulation of α-ketoglutarate and pyruvate was found in ammonia-intoxicated brains [167]. Nearly twenty years later, hyperammonia exposure was demonstrated to inhibit αKGDH in brain preparations [168, 169]. Years later, with advanced techniques (such as NMR), the inhibition of aKGDH by ammonia was confirmed to be severe enough to directly cause cerebral lactate accumulation in ALF [157], even with adequate oxygen supply.

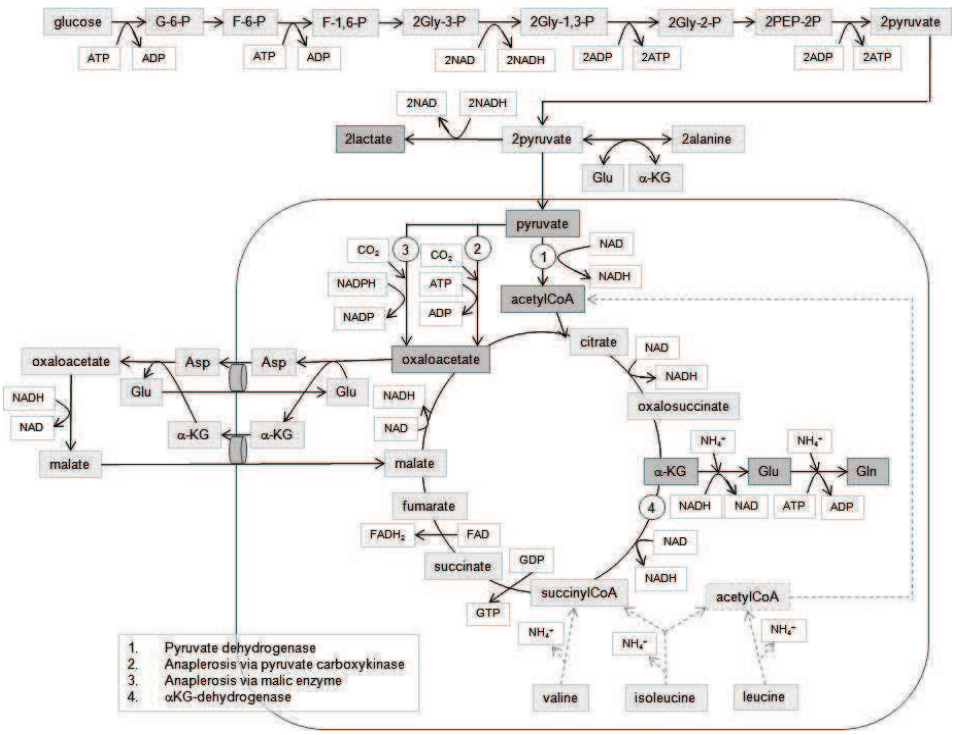

Figure 2. Glucose metabolic pathway. The mitochondria is separated from the outside cytosol. Glu, glutamate; Gln, glutamine; α-KG, alpha-ketoglutarate; Asp: aspartate.

3.2.3 Two direct consequences of aKGDH inhibition by ammonia

As increased cerebral ammonia in ALF [26, 170, 171] inhibits aKGDH [168], two important alterations result: 1) stimulates aKG-glutamate-glutamine metabolic pathway, 2) reduced energy (ATP) production.

In support of the first change, increased cerebral aKG has been demonstrated in ammonia-infused animals [172-174], and in HE patients [175, 176]. In turn, high aKG induces glutamate production by glutamate dehydrogenase (GDH) in both astrocytes and neurons (the NH-group is derived from amino acids and not from free ammonia in this step). Subsequently, glutamate incorporates free ammonia to generate glutamine by GS in astrocytes, which is important for ammonia detoxification in the brain [177, 178]. Both the inhibition of aKGDH by ammonia and the increased glutamine synthesis by ammonia redirects the TCA cycle and stimulates the aKG-glutamate-glutamine metabolic pathway. When using NMR, increased PC metabolic flux, in association with increased glutamine de novo synthesis, has been demonstrated in the brain of hyperammonemic animals [179-181], ischemic ALF animals [114, 115], and ammonia-exposed cultured astrocytes [182, 183].

In viewing the second change, as the TCA cycle is responsible for energy (ATP) production, inhibited aKGDH results in impaired energy production in association with increased PC flux and decreased pyruvate dehydrogenase (PDH) flux, in ALF.

In normal subjects, astrocytes account for ~5% of glucose-derived ATP production in the brain, and neurons account for ~95% of glucose-derived ATP production in the brain [184, 185]. Obviously, neurons contribute much more than astrocytes for energy production in the brain. Based on this knowledge, decreased PDH flux more closely reflects decreased ATP production in neurons or in the whole brain, and increased PC flux reflects increased "loss of glucose for ATP production in astrocytes". PDH flux is significantly decreased in the brain of coma stage HE animals [114, 115], which indicates an impaired neuronal energy supply at the late/severe condition of HE. Ammonia-inhibited aKGDH as well as ammonia-inhibited PDH activity [115] may account for reduced PDH flux. On the other hand, the "loss of glucose for ATP production in astrocytes" is evident by an increase of astrocytic glucose utilization for PC flux from 30% in normal conditions [186] to 80% in hyperammonia situations [115, 181, 183, 187]. These findings of alterd PDH and PC flux support the view of a potentially impaired energy (ATP) generation in brain in hyperammonemic HE, which is more substantial at coma stage HE.

3.2.4 Increased glucose utilization and lactate accumulation in ALF

Increased glucose utilization has been demonstrated in hyperammonic conditions, which is believed to be a compensatory response to impending decreased ATP production (blocked glucose metabolic flux) due to ammonia-induced aKGDH inhibition. In support of this notion, increased cerebral glucose metabolism has been described in ammonia-adminstrated animals [188-191], which may attribute to increased endothelial/astrocytic glucose transporter GLUT-1 [192, 193], or increased cerebral phosphofructokinase activity [194-196] under hyperammonia stimulation. All these alterations will at least increase the production of NADH and ATP in cytosol.

Increased cerebral lactate accumulation in ALF, which reflects a decreased cerebral pyruvate oxidation, is believed to be another consequence of ammonia-induced aKGDH inhibition. Increased cerebral lactate has been consistently described in various animal models of ALF [31, 112, 113, 115, 154-156, 197-200], as well as in ALF patients [157-159], which correlates well with the worsening of HE and cerebral edema in ALF [114, 154, 163, 197]. In ALF, the cerebral oxygen supply/delivery only slightly decreased in early course, whereas it increased in the late advanced stages of ALF [39, 157, 201], a phenomenon that parallels the increase of cerebral blood flow (CBF). On the other hand, cerebral oxygen consumption is decreased [39, 157, 201, 202]. When comparing results from comatose [39] to early stage [202] ALF patients, cerebral glucose consumption increased 3 times, whereas cerebral oxygen consumption decreased 5.2 times. Therefore, hypoxia could not be used to explain cerebral lactate accumulation in ALF, which is more likely due to aKGDH inhibition by hyperammonia.

3.3 Astrocyte metabolism and function in ALF

Astrocytes account for one third of the brain volume, which keeps the homeostasis of extracellular environment for normal neuronal function. Evidence of altered astrocytic function is substantial in HE. In relating to its ammonia detoxification [67], astrocytic neuropathologic changes are the features of ALF [17, 203], with altered astrocytic protein function [67, 203-206].

GFAP is an astrocytic intermediate filament protein [207]. Decreased GFAP expression has been demonstrated in the brain of ischemic ALF animals [208] and ammonia-treated cultured astrocytes [208, 209]. The reduction of GFAP is selective, whereas another astrocytic filament protein S-100β is unaltered in these conditions. A decrease in GFAP may compromise visco-elastic function and facilitate the astrocyte swelling.

GS is a cytoplasmic enzyme, largely localized in cerebral astrocytes, which functions to detoxify ammonia in the brain. In hyperammoneamic conditions, increased cerebral glutamine is observed [63, 65, 210]. However, cerebral GS expression and activity is unaltered or decreased [55, 70, 211-213].

Decreased glycine transporter (GLYT1) expression (an astrocytic glycine transporter) has been observed in the brain of ischemic ALF animals [214] and ammonia-stimulated cultured-astrocytes. Accordingly, increased cerebral extracellular glycine is demonstrated in ALF animals at coma stage [113]. Glycine

could function as a positive allosteric modulator of the NMDA receptor, with the potential to lead to increased glutamatergic neurotransmission in HE.

The “Peripheral-type” benzodiazepine receptor (PTBR) is localized on the outer mitochondrial membrane of glia and neurons. PTBR consists of three subunits: 1) 18 kDa isoquinoline binding protein (IBP), 2) 34 kDa voltage-dependent anion channel (VDAC), 3) 30 kDa adenine nucleotide carrier (ANC) [215]. Increased IBP mRNA or protein expression have been described in the brain from deceased HE patients [216], and ischemic ALF rats [217]. In addition, increased PTBR ligand PK11195 binding has been demonstrated in autopsied brain samples from HE patients [218], in the brains of animals with thioacetamide (TAA)-induced ALF [219] and ischemic ALF animals [220]. The observed increase of PTBR (PK11195) binding correlates with clinical severity of HE [66, 218, 221, 222]. Evidence in support of the notion that hyperammonia upregulates PTBR was provided by increased IBP expression or PK11195 binding in the brain from ALF patients and animals (see above), as well as in the brain of ammonia-injected animals [219], in the brains of animals with congenital urea cycle deficit [223], and in ammonia-exposed cultured astrocytes [224].

GLUT-1 is one of the cerebral glucose transporters, expressed in astrocytes and endothelial cells, responsible for the uptake of glucose into the brain. Increased cerebral GLUT-1 expression has been observed in experimental ALF [192], which correlates the increasing cerebral lactate accumulation and cerebral edema [115]. On the other hand, the neuronal glucose transporter (GLUT-3) is unaltered in the brain in ALF. Increased

GLUT1 expression is considered as a compensatory regulation associated with increased glycolysis due to inhibited cerebral TCA by ammonia in ALF. Elevated GLUT1 expression may also contribute to increased inward movement of water into the glial cerebrovascular endothelial cells in ALF [225].

Altered cerebral aquaporin IV expression (astrocytic water channel protein) has been observed in experimental ALF [226-228]. Aquaporin IV has been shown to facilitate cell swelling in in vivo [229] and in vitro [142] experiments. The down-regulation of cerebral aquaporin IV in ischemic ALF animals may reflect its compensatory adjustment (unpublished data).

3.4 Synaptic transmission (electrophysiology)

Suppression of cerebral excitatory synaptic transmissions by ammonia has been demonstrated both in in vivo [230, 231] and in vitro [232-235] experimental settings. Presynaptically, ammonia may decrease glutamate release from nerve terminals by (1) either reducing its synthesis from glutamine [236] or by (2) blocking the transmission of the action potential to nerve terminal [231]. Postsynaptically, ammonia may (3) impair the interaction between glutamate and glutamate receptors [234]. The suppression of the inhibitory postsynaptic potential (IPSP) by ammonia has been evident in in vitro [237, 238] and in vivo [239, 240] experiments. Its mechanism involves the inhibition of Cl^- extrusion from neurons by ammonia [239, 240], which subsequently prohibits the opening of Cl^- channels by the inhibitory neurotransmitter (for example, GABA).

3.5 Changes in cerebral neurotransmission

Alteration of various neurotransmitter systems have been proposed to induce neurologic dysfunction in HE [241], which includes impaired synaptic transmission (discussed above), alteration of the glutamate system, serotonin system, and norepinephrine system.

3.5.1 Glutamate system in ALF

3.5.1.1 Intracellular brain glutamate (whole brain glutamate)

Decreased cerebral glutamate levels have been demonstrated in ammonia-injected rats [105, 242], ischemic ALF animals [31, 52], thioacetamide-injected ALF animals [52, 156] and hepatectomized animals [243]. Three possible mechanisms are proposed: 1) The increased cerebral ammonia, rising from normal 0.05-0.1mM to 1-5mM in ALF [31], activates its detoxification reaction to generate glutamine from glutamate, mainly by astrocytic GS, which leads to low glutamate and high glutamine in the brain [114, 115, 187]. 2) Hyperammonia could also reduce glutamate content in cultured neurons [244], which may relate to ammonia's inhibitory effect on PAG [245]. 3) The reduced cerebral glutamate partially attributed to reduced de novo synthesis from glucose in neurons [115]. As a result, reduced cerebral glutamate results in decreased ammonia removal in

astrocytes [211, 246], impaired excitation/inhibition in the nerve terminals [247], and altered energy metabolism in multiple cells [248].

3.5.1.2 Extracellular brain glutamate

The whole brain glutamate concentration (mM) mainly reflects its intracellular levels (neuronal intracellular glutamate: ~10mM; glial intracellular glutamate: ~50μM). Extracellular glutamate are very low (μM) (CSF glutamate: ~1μM) to give a good signal-to-noise ratio. An increase of cerebral extracellular glutamate concentrations (~3μM) has been demonstrated in hepatic toxin-induced ALF animals [52, 249, 250], ischemic ALF animals [52, 251, 252], ammonia-injected animals [253], and in ALF patients [158]. Increased extracellular glutamate in brain parallels the worsening of HE and cerebral edema in ALF animals [113, 254] and ALF patients [158].

A decreased glutamate uptake was proposed as a possible mechanism to explain increased cerebral extracellular glutamate accumulation in ALF. Evidence in support of this view is provided by brain preparations from thioacetamide-injected animals [255], ammonia-treated neuronal preparations [256], ammonia-exposed cultured astrocytes [257]. Anatomically, astrocytic glutamate transporters EAAT-1 and EAAT-2 are considered most responsible for glutamate clearance [258]. Down-regulation of EAAT1 expression has been observed in ammonia-exposed cultured astrocytes [259, 260], which accompanies decreased L-glutamate [257] or D-aspartate [259, 261] uptake. Decreased EAAT2 expression has been described in the brain of ischemic ALF animals [227, 262]

and thioacetamide-injected ALF animals [263], which accompanies reduced D-aspartate uptake from these animals' brain preparations [264, 265], and elevated extracellular brain glutamate [266]. These findings support the view that ammonia-induced down-regulation of EAAT1 and EAAT2 are the cause of increased extracellular glutamate in brain in ALF.

In addition, increased glutamate release from cultured astrocytes has been demonstrated in ammonia-stimulation [267], hypoosmotic-conditioning [268], or exocytotic activation [269, 270], which relates to a pH-mediated, Ca^{2+}-dependent mechanism [270, 271]. Increased glutamate release gives a second mechanism to explain the increased extracellular glutamate level in HE associated with hyperammonia.

3.5.2 Serotonin system in ALF

An activation of cerebral serotonin system is evident in ALF. Increased cerebral 5-HT or the 5-HT metabolite (5-HIAA) has been described in animals with thioacetamide-induced ALF and ischemic ALF [272, 273], and in ALF patients [274, 275]. Decreased expression of cerebral serotonin transporters was reported in ALF rats [276]. In addition, a down-regulation of cerebral serotonin 5-HT2 receptors has been demonstrated in thioacetamide-induced ALF [277] and in hyperammonemic animals [278], which is considered as a compensatory response. Moreover, administration of a nonselective 5-HT antagonist (methysergide) improves locomotive activity in TAA-induced ALF animals [279].

3.5.3 Noradrenaline in ALF

The neurotransmitter noradrenaline is synthesized from dopamine by dopamine β-hydroxylase. Studies of the noradrenergic systems have been mainly undertaken in ALF. (1) Reduced cerebral noradrenaline has been reported in animals with hepatectomy [280], ischemic ALF [281], and thioacetamide-induced ALF [272]. (2) An increase of cerebral extracellular noradrenaline has been demonstrated in hepatectomized animals [282], and in ischemic ALF animals [266], which is considered related to the down-regulated cerebral noradrenaline transporter [283]. (3) Decreased cerebral noradrenaline α and β receptor binding sites were also described in ALF rats [266]. Whether these findings represent a noradrenaline over-stimulation in ALF waits further investigation.

3.6 Osmolytes

3.6.1 Organic osmolytes in ALF

Cerebral osmolytes (glutamine, alanine, myo-inositol, taurine) are altered in ALF. The consistent findings of several-fold elevated brain glutamine in ALF support the role of glutamine in the pathogenesis of astrocyte swelling (which has been described in the previous section: glutamine-osmolyte-edema hypothesis). Alanine is another recognized organic osmolyte. Increased cerebral alanine has been reported in

experimental ALF [31, 115], and the elevation correlates well with the progression of HE and brain edema [115]. A special feature of cerebral astrocytes is its capability to adapt against cell-swelling by reducing some of the organic osmolytes (myo-inosital and taurine) [284, 285]. In the clinical and experimental setting of hyperammonemia, increased cerebral glutamine accumulation is often associated with a slight decrease of myo-inositol [182, 286]. The reduced myo-inosital is believed to exert a compensatory role in the brain of ALF in maintaining an osmotic homeostasis. In addition, a reduction of taurine (another astrocytic intracellular osmolyte) was also observed in both in vitro [287] and in vivo [124, 288, 289] experimental setting of hyperammonia, which indicates a volume regulatory role of taurine.

3.6.2 Inorganic osmolytes in ALF

Sodium is a very important inorganic osmolyte in the body. The incidence of hyponatremia is approximately 25-33% in ALF patients with severe HE [37, 290, 291]. Clinically, acute hyponatremia can induce brain edema directly [292]. In one experimental study, hyponatremia significantly exacerbated ammonia-induced cerebral edema in rats [293].

3.7. The role of inflammation in the pathogenesis of HE and brain edema in ALF

The role of infection and inflammation in the development of ALF has been recently proposed and the mediators of infection and inflammation might have a synergistic

effect with ammonia, which will exacerbate and accelerate the pathophysiologic change of ALF [294, 295]. In fact, infection and inflammation are common features in ALF's natural history. Infection is documented in at least 80% of ALF patients [296], which leads to a rise of proinflammatory cytokines (TNF-α, IL-6 and IL-1β) in the blood [297-300]. Besides coexisting infection, ALF itself is an inflammatory state, in which increased pro-inflammatory cytokine levels are observed [298, 301, 302], most obvious in patients with elevated ICP. The production of systematic pro-inflammatory cytokines might be attributed to stimulation of the immune system by infection or mediators released from necrotic liver, or a direct release of cytokines from the necrotic liver.

In two large clinical studies of ALF, retrospective [303] or prospective [304], the appearance of SIRS and/or infection is correlated with the severity of HE, increased ICP and higher mortality rate. The results of these two studies support the view that inflammation plays a role in the development of brain edema and HE in ALF [305]. In another case report of an ALF patient with uncontrolled high ICP, hepatectomy decreased ICP and produced hemodynamic stability [306] associated with a reduction of circulating TNF-α, IL-6 and IL-1β [306], without alteration in arterial ammonia levels. This single case provides evidence that necrotic liver-derived systematic proinflammatory cytokines might exacerbate brain edema in ALF [306]. In the case of acute-on-chronic liver failure, inflammation and infection may be important contributing mechanisms [307]. Similarly, the synergistic effect of inflammation on ammonia toxicity has been demonstrated in many chronic liver

diseases [295, 308, 309]. Intestinal-derived bacteria passing into lymph nodes and further into blood provide a possible pathogenic route [310]. Moderate hypothermia effectively controls increased ICP in ALF patients [311]. Although multiple beneficial effects have been demonstrated by hypothermia treatment, including reduction of arterial ammonia, its capability to decrease pro-inflammatory cytokines in blood is also a possible mechanism [311].

In a pig ALF model induced by hepatotoxin-amatoxin, co-treatment with LPS worsens and accelerates the progress of brain edema and HE [312]. The findings from this study indicate that both necrotic liver and infection could produce pro-inflammatory cytokines. Levels of inflammation correlated with the pathogenesis of HE and brain edema in ALF. Clinical and experimental evidence supports the concept that there is a synergistic effect between hyperammonia and inflammation in the pathogenesis of HE and brain edema in ALF.

3.8 CBF in ALF

3.8.1 Clinical and experimental observations

In general, increased CBF is observed in ALF patients with high ICP [157, 313-316]. In a clinical study, increased CBF has been shown to precede the development of high ICP [317]. An impaired CBF autoregulation has also been described in ALF patients, as well as abnormal response to hypercapnia [318] and norepinephrine [319, 320]

challenges, which represent a state of vasodilatation. Increased CBF and cerebral vasodilatation state, observed in ALF, bring out a concept of “luxury cerebral perfusion”. In a rat model of portacaval-shunt followed by ammonia infusion [121], a rise of CBF has been observed preceding the development of high ICP and brain herniation [93]. In this model, increased CBF is positively correlated with brain edema [25, 321].

3.8.2. Possible mechanisms to induce increased CBF in ALF

Evidence linking glutamine to high CBF and loss of cerebrovascular autoregulation is provided by MSO treatment. Administration of MSO (a GS inhibitor) to hyperammonemic animals prevents the accumulation of glutamine, the increase in CBF and development of cerebral edema [93], and restores cerebrovascular auto-regulation [94, 322, 323].

Reactive oxygen and nitrogen species have been shown to mediate cerebral vasodilatation from different causes [324]. Increased cerebral nitric oxide (NO) (an important vasodilator) has been demonstrated in ammonia-infused rats, accompanying an increased CBF [93]. MSO prevents both high CBF and cerebral NO production in these rats.

Heme oxygenase catalyzes the degradation of heme to generate carbon monoxide (CO), which is involved in vascular muscle relaxation. Hyperammonia-induced upregulation of HO-1 mRNA and protein expression has been demonstrated in the brain

of ALF animals [325]. Administration of zinc-protoporphyrin II (a non-selective HO-1,2,3 inhibitor) to ammonia-infused rats, prevented the occurrence of high CBF and brain edema [200, 326].

Increased SIRS grading and circulating cytokines have been shown in association with high CBF and brain edema development in ALF [294, 311]. The use of hepatectomy to one ALF patient, which decreases circulating pro-inflammatory cytokines, leads to significantly reduced CBF and ICP [306]. Moreover, the addition of LPS to ammonia-infused rats produced more severe levels of high CBF and ICP than either LPS administration or ammonia-intoxication alone [327].

Activation of potassium channels in vascular smooth muscle induces vascular smooth muscle relaxation. It has been demonstrated that increased extracellular potassium leads to potassium channel opening [328], and cerebral vessel relaxation [329]. Increased cerebral extracellular potassium has been reported in ALF patients [320, 330] and in ammonia-infused rats [331].

The involvement of the arachidonic acid pathway in regulating CBF has been reported previously [332, 333]. Administration of indomethacin (a COX-2 inhibitor) to ammonia-infused rats prohibits the rise of high CBF and cerebral edema in these animals [24]. This data indicates the possible involvement of prostaglandins in mediating high CBF and ICP in ALF.

3.8.3 Mechanisms of increased CBF in producing cerebral edema/high ICP in ALF

Three potential mechanisms have been proposed to explain the influence of high CBF on the generation of cerebral edema and high ICP in ALF [334]: (1) high CBF induces high hydrostatic pressure on BBB, which facilitates water movement across the BBB into the brain; (2) high CBF enhances the cerebral exposure to blood-born toxic factors (such as: ammonia, cytokines), which facilitate detrimental effects directly or via their uptake by the brain; (3) high CBF increases cerebral blood volume, which increase ICP by itself.

3.9 Alterations in BBB in ALF

In ALF, BBB is structurally intact [17, 18, 21]. This view is further confirmed by a recent MRI study in ALF patients [27]. However, morphologic changes of endothelial cells (an increased vacuolization and pinocytosis) have been observed in ALF [17, 18, 20, 335]. In both clinical and experimental HE, BBB-related functional disturbances have been observed. An increased expression of GLUT1 (the BBB glucose transporter) was observed in rats with ischemic ALF [193], which presumably indicates an enhanced water movement into the brain [336]. Altered expression of other cerebral endothelial genes, such as eNOS, occludin, VCAM, AQP4 have also been reported in experimental ALF [337]. Although the BBB remains impermeable to large molecular weight molecules, increased permeability of the BBB to small molecules such as inulin [338], sucrose [338], GABA [339, 340], Trypan blue [22, 341]

or horseradish peroxidase [19] has been demonstrated in various experimental animal models of ALF.

Elevated circulating ammonia has been hypothesized to trigger functional change of permeability, despite an anatomically intact BBB [93, 342, 343]. Inflammation is another factor which can cause BBB alterations [295, 327, 344-346].

3.10 Neuroinflammation in ALF: role of microglia

Microglia comprise nearly 10% of the cerebral cellular population, which function as resident mononuclear phagocytes of the brain [347, 348] and give differential response to various stimuli [349]. Microglia share lineage-related phenotypes with bone-marrow-derived monocytes and macrophages [350]. As macrophages, activated microglia could eliminate deleterious debris by phagocytosis and support tissue survival by secreting neurotrophic factors at cerebral injury sites. Microglia are considered to be major cell types in CNS responsible for cytokine production [351-355]. It has been shown that activated microglia can generate various cytotoxic substances in *in vitro* settings, its release has been implicated in the development of several neuroinflammatory/neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, AIDS dementia, multiple sclerosis, amyotrophic lateral sclerosis, traumatic brain injury [356], stroke [357], and Wernicke's encephalopathy [358].

It has been proposed that activated microglia and related inflammatory responses are detrimental to the brain. In *in vitro* settings, microglia have been demonstrated to be able to produce several cytotoxic substances, such as ROS [359, 360], NO, proteases, arachidonic-acid derivates, glutamate [361], quinolinic acid [361], TNF-a, IL-1b [362-366]. These factors influence the CNS cellular system in an autocrine and/or a paracrine fashion. For instance, it has been suggested that microglia-generated TNF could induce RNOS production from astrocytes [367, 368]. Results of the studies described in this thesis will provide the first direct evidence for a role of microglial activation in the pathogenesis of the encephalopathy and brain edema in ALF.

4. Clinical Features of ALF

ALF is defined as the appearance of HE and acute deterioration of liver function in patients without any previous history of liver dysfunction. Encephalopathy is seen in almost all cases of ALF [11, 291, 369]. Neuro-psychiatric disturbances in ALF develop in sequence from insomnia, lack of concentration, to confusion, disorientation, coexisting with agitation and manic behaviour, before developing into stupor and coma. Neuro-psychiatric deterioration in ALF can proceed into deep coma rapidly in a few hours.

Using continuous EEG monitoring, subclinical seizures occur in 45% of ALF patients with severe HE [370]. Two Indian groups reported a 22-25% frequency of seizure in ALF patients [37, 290]. The frequency of seizures is positively associated with arterial

ammonia levels [37].

Brain edema is the most serious complication of ALF, seen in 35-50% of ALF patients with grade III HE, 50-80% of ALF patients with grade IV HE [371-373]. Cerebral edema can lead to increased ICP, which results in brain herniation and death [374-376]. In clinical studies, 42% - 75% of deaths in ALF are attributable to increased ICP [377]. Multiple factors have been considered to contribute to the development of brain edema [204, 378, 379], of which hyperammonia is most important [26].

The cranium has a fixed volume with very little room for adaptation. Three compartments define its volume: 80-90% of parenchyma, 10% of intravascular blood and 3-5% of CSF. In the parenchyma, extracellular fluid accounts for 10-20% of the volume, astrocytes account for 30% of total cells [321]. In ALF, astrocyte swelling [380], cerebral vasodilation, and increased CBF collectively contribute to the elevation of intracranial hypertension. In ALF, brain edema develops rapidly, which leaves little time to compensate resulting in increased ICP.

4.1. Prognosis of ALF

ALF with stage I and II HE is associated with a spontaneous recovery rate of 70%, whereas stage III and IV HE is associated with a < 20% spontaneous liver recovery [381]. Survival rate without transplantation is highest in acetaminophen related cases (68%) and lowest in cases related to idiosyncratic drug reactions (25%)

[382]. The overall survival rates have improved from 20-40% in pre-transplant period to more than 60% when liver transplantation technique was developed [37, 382]. King's college criteria are considered most accurate and are widely used [383, 384] in the prediction of the survival and the need for liver transplantation in ALF patients (see details in liver transplantation section).

5. Treatment of HE type A, in ALF patients

5.1 Initial practical steps to follow, in ALF patients

Patient's head should be kept up at a 20 degree angle to encourage venous outflow and decrease in ICP [385]. Cerebral perfusion pressure (CPP = mean arterial pressure – ICP) must be kept between 50 to 65 mmHg to avoid cerebral hypoperfusion and hyperhemia [386, 387]. Vasopressin and its analogues should be used with extreme caution, because they may worsen hyperammonemia and increase ICP by elevating CBF [316, 388-390]. Spontaneous hypoglycemia must be corrected. Other factors that could increase ICP, which must be monitored and corrected, include hyperthermia [372, 391, 392], hyperglycemia [392], hyponatremia [293, 393], and hypercapnia [394]. The acid/base balance should be monitored closely, since hyperlactatemia can worsen cerebral hyperemia [115, 158]. Volume extension (with a mix of cystalloids and colloids) is needed for most of the patients. Up to 80% of ALF patients complicated with high ICP require extracorporeal renal support [395]. The situation of the patient

should be discussed with the liver transplantation unit ASAP to determine candidacy for liver transplantation.

5.2 Treatments aimed at reducing ammonia levels

Recent studies have not demonstrated any beneficial effects of lactulose administration in ALF patients [396, 397]. In ischemic ALF animals, LOLA prevents the development of brain edema [32], by increasing muscle glutamine synthesis [48, 398], which results in a reduction of blood ammonia.

5.3 Treatment aimed at the decrease of cerebral edema and ICP

Mannitol is a well accepted classic treatment in controlling increased ICP, whose mechanisms relate to an increase of osmolality in cerebral capillaries, which results in water extraction from the brain, and water excretion by the kidney. Its beneficial effect in controlling high ICP has been clearly demonstrated in ALF patients [399]. Hypertonic saline (30%) is used as a prophylactic measure to prevent brain edema in ALF [400].

5.4 Treatment aimed at reducing CBF

Mechanical hyperventilation which induces arterial hypocapnia ($PaCO_2$, 3.5-5 kPa) could result in a short-term vasoconstriction and a decrease in CBF [401]. Propofol, a

short-acting sedative agent, reduces CBF through metabolic suppression, which demonstrates beneficial effects in reducing ICP in ALF patients [402, 403]. Indomethacin, a cyclo-oxygenase inhibitor [404], induces cerebral vasoconstriction and CBF reduction, which decreases ICP in ALF patients [315, 405], and prevents cerebral edema in ALF animals [24]. Thiopental Sodium, an ultra-short acting barbiturate, has vasoconstructive effects, and should be used only as a last resort in ALF patients [406].

5.5 Treatment aimed at neurotransmission systems

Memantine is a NMDA-R inhibitor, which has been shown to improve neurologic symptoms and/or prevent brain edema in experimental animals with toxic or ischemic liver failure [407]. Administration of phenytoin, a sodium channel blocker, to ALF patients did not result in the improvement of subclinical seizures, brain edema or high ICP [290, 370]. Benzodiazepine partial inverse agonists (Rol5-4513 or sarmazenil) are mild antagonists of GABA-A receptor [408], which has been shown to improve HE symptoms in ALF animals [409, 410], probably attributed to its interaction with GABA-agonist neurosteroids [411].

5.6 Treatment aimed at reducing systemic inflammation

Treatment with N-acetylcysteine (NAC) in acetaminophen-induced ALF patients significantly enhances their survival rate [412-414], by improving glutathione reserves and addition of NAC to standard therapy significantly improves ALF patient survival

from non-acetaminophen causes [415, 416]. A relatively old study showed that dexamethasone did not improve survival in ALF patients [399]. Several studies showed that prophylactic antibiotics decreased infection rate ($P < 0.05$), but did not change mortality in ALF patients [417-419]. For practical purposes, empirical wide-spectrum antibiotics should be administered to ALF patients with progressive HE. Hepatectomy, which decreases systemic inflammation, is only recommended in ALF patient on the transplant wait list as a last approach, when all other treatments fail [306, 420, 421].

5.7 Mild Hypothermia: a treatment involving multiple mechanisms

The beneficial effects of mild hypothermia (32-35°C) in the control of high ICP in ALF patients have been demonstrated in clinical studies [39, 311, 314, 377, 422, 423]. Mild hypothermia effects multiple mechanisms in ALF, which include: (1) decreased cerebral and arterial ammonia levels [39, 311, 424-427], (2) prevent cerebral osmolyte accumulation [114, 124], (3) prevent extracellular glutamate and lactate accumulation in the brain [197, 254, 428], (4) restore cerebro-vascular autoregulation and decrease CBF [121, 314], (5) improve cerebral glucose metabolism [39, 114, 311], (6) attenuate systemic and cerebral inflammation [311, 314], and (7) improve cardiovascular dysfunction [311].

5.8 Liver transplantation

Liver transplantation is currently the only radical solution for ALF. Survival after OLT (orthotopic liver transplantation) is similar in the Far East and the West (60-80%), which is much higher than those who do not receive a graft (15-20%) [429, 430]. In both acetaminophen-induced ALF [431-433] and non-acetaminophen-related ALF [432, 434, 435], King's College criteria give more predictive values and accuracy than Clichy criteria.

5.9 Artifical and bio-artifical liver support systems

"High-volume plasmapheresis" has been used in successful reduction of ammonia, and improvement in mental state, hemodynamics, and cerebral perfusion [45, 436]. Molecular Adsorbent Recirculating System (MARS) [437] has been shown to be effective in the improvement of HE symptoms, reduction of ammonia, bilirubin, creatinine, and aromatic amino acids in ALF patients [438], and attenuates cerebral edema and high ICP in ALF animals and patients [439, 440].

Regarding bioartificial liver support systems, about 10^{10} hepatocytes are needed to represent 10-30% of the liver mass in fulfilling its synthetic, detoxifying, and excretory functions [441]. The ELAD system is beneficial in attenuating HE symptoms, ICP, and haemodynamic abnormalities, but has no effect on survival in ALF patients [442]. The bioartificial liver device (BAL) system reduces ICP and improves blood parameters (such as glucose, ammonia, and bilirubin) [443], and significantly increased survival in a subgroup of patients with ALF [444].

6. Summary

HE comprises a spectrum of neuropsychiatric abnormalities observed in ALF patients. A quick worsening of consciousness and increasing progress of cerebral edema, which leads to high ICP, cerebral herniation and death, are characteristics of ALF. Multiple factors are found responsible for the development of HE in ALF, whereas hyperammonemia (which can rise to millimolar concentrations at coma stages) is considered the most crucial factor in defining the pathogenesis of HE in ALF.

Body ammonia is mainly produced from (1) intestines, through the breakdown of amino acids and urea, and (2) kidneys, via the degradation of glutamine. The rate of ammonia generation by intestines is not altered in ALF, whereas kidney shifts into ammonia excretory status during hyperammonemia. In ALF, decreased periportal urea synthesis and reduced perivenous glutamine synthesis lead to impaired ammonia detoxification and hyperammonemic state. Muscle and brain glutamine synthesis are both important extra-hepatic pathway for ammonia removal in ALF. In the brain, the astrocyte is the main cellular type for ammonia removal, by the conversion of glutamate into glutamine. In ALF, increased cerebral ammonia leads to glutamine accumulation in astrocytes, which is responsible for the development of cerebral edema in ALF. The underlying mechanisms have been explained by two mutual completing hypotheses: “glutamine-osmolyte-edema hypothesis” and “glutamine-RNOS-edema hypthesis”.

Ammonia at millimolar concentration induces multiple system dysfunctions, including a direct inhibitory effect on both CNS synaptic excitation and inhibition, and an increase of cerebral tryptophan uptake by glutamine-tryptophan exchange, which results in increased synthesis of serotonin and metabolites in the brain. In addition, altered cerebral glucose metabolism in ALF has been attributed to hyperammonemia, with altered TCA cycle flux and cerebral lactate accumulation. Furthermore, altered astrocytic protein expression (including GFAP, the glutamate transporter, the glycine transporters, and PTBR) have been demonstrated in ALF. These changes are associated with astrocytic morphologic and functional changes. Other toxic substances may act synergistically with ammonia in ALF.

Alterations of neurotransmitter systems have been demonstrated in ALF, which include increased extracellular glutamate, elevated NMDA receptor activation, and altered serotonin and noradrenaline systems. Recently, systemic inflammatory response has been demonstrated contributing significantly to the development of HE in ALF. Additional factors, such as increased CBF and altered BBB function, should also be taken into account for the pathogenesis of HE in ALF.

During the therapeutic management of HE in ALF, the treatment of precipitating factors, HE itself, the failing liver, and multi-organ systems are needed. Reducing ammonia production and increasing ammonia removal are traditional strategies in the treatment of HE. In ALF, therapeutic interventions in an attempt to prevent and treat impending cerebral edema and high ICP have been developed, which include mannitol,

hypertonic saline, prophylactic antibiotics, NAC, hemofiltration, hyperventilation, etc. In addition, therapeutic approaches aimed at modulating neurotransmitter systems have been designed and showed beneficial effects in ALF, which includes: NMDA receptor antagonist (memantine), and benzodiazepine receptor inverse agonists. Mild/moderate hypothermia is a promising new approach in the improvement of HE and cerebral edema in ALF, and involves multiple mechanisms, including reducing blood and brain ammonia, enhancing cerebral glucose oxidation, ameliorating systematic inflammation, etc. Liver transplantation is an efficent and radical approach in prolonging the survival of ALF patients, which provides up to 90% survival rate in post-transplantation populations. With the increasing understanding of the pathophysiology of HE in ALF and the advance in new technologies, more effective new therapeutic interventions will be created.

7. Objectives of the proposed studies

The objectives of the studies now proposed are, using a well-validated animal model of ALF characterized by liver devascularization, hyperammonemia and brain edema, the following:

1. To demonstrate that neuroinflammation characterized by microglial activation and increased proinflammatory cytokines occurs in ALF

2. To demonstrate that mild hypothermia attenuates neuroinflammation and brain edema in ALF

3. To investigate the effect of minocycline, an antibiotic shown in previous unrelated studies to reduce microglial activation, attenuates brain proinflammatory cytokines and brain edema

4. To evaluate the benefit of mild hypothermia and minocycline on oxidative/nitrosative stress taken in relation to the attenuation of brain edema

Proclaim:

I contributed to the design, experiment, statistical analysis and production of the 4 articles included in this thesis.

Dr. Butterworth and Dr. Desjardins supervised, and helped design and revise the following 4 articles.

CHAPTER 2

STUDIES ON THE PATHOGENESIS AND NEW THERAPEUTIC INTERVENTION OF HE.

Article 1:

Direct evidence for central proinflammatory mechanisms in rats with experimental acute liver failure: protective effect of hypothermia

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Abstract

It has been proposed that proinflammatory mechanisms are involved in the pathogenesis of brain edema in acute liver failure (ALF). The aim of this study was to assess the contribution of cerebral inflammation to the neurologic complications of ALF and to assess the antiinflammatory effect of mild hypothermia. Upregulation of CD11b/c immunoreactivity, consistent with microglial activation, was observed in the brains of ALF rats at coma stages of encephalopathy. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) mRNAs were increased two to threefold in the brains of ALF rats compared with that in sham-operated controls. The magnitude of increased expression of proinflammatory cytokines in the brain was correlated with the progression of encephalopathy and the onset of brain edema. Significant increases in IL-1β, IL-6, and TNF-α levels were also found in the sera and cerebrospinal fluid (CSF) of these animals. Mild hypothermia delayed the onset of encephalopathy, prevented brain edema, and concomitantly attenuated plasma, brain, and CSF proinflammatory cytokines. These results show that experimental ALF leads to increases in brain production of proinflammatory cytokines, and afford the first direct evidence that central inflammatory mechanisms play a role in the pathogenesis of the cerebral complications of ALF. Antiinflammatory agents could be beneficial in the management of these complications.

Keywords: acute liver failure; brain edema; hepatic devascularization; hypothermia; microglial activation; proinflammatory cytokines

Introduction

ALF that results from viral infection or toxic liver injury is a life-threatening condition. HE and brain edema are serious neurologic complications of ALF, and their appearance heralds the need for emergency liver transplantation [380]. Several theories have been proposed to explain the pathogenesis of HE and brain edema, among which ammonia toxicity has received the most attention [204, 378]. However, ammonia is not the only pathophysiologic entity with the potential to adversely affect cerebral function in ALF. In particular, there is evidence that inflammation may also play a significant role. For example, large clinical studies have convincingly shown a higher prevalence of the ‘‘systemic inflammatory response syndrome’’ in ALF patients [303]. Systemic inflammatory response syndrome is a response to the presence of proinflammatory cytokines, such as the interleukins, IL-1β and IL-6, as well as to tumor necrosis factor-α (TNF-α), and increased circulating levels of cytokines have been reported consistently in ALF patients [298, 299, 314, 445]. Cytokines may also be formed and released by the necrotic liver. Studies on arteriovenous difference by Jalan et al (2003) suggest a net production of proinflammatory cytokines in the brains of patients with ALF [314].

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However, direct evidence for a role of increased brain cytokines in the pathogenesis

of the cerebral complications of ALF is still lacking. Therefore, the aim of this study was to measure TNF-α, IL-1β, and IL-6 mRNA, and protein expression as a function of neurologic status in the brains of rats with ALF because of hepatic devascularization. These cytokines were chosen in view of earlier reports of their selective increase in both experimental and human ALF (above). In addition, given the recent upsurge of interest in hypothermia in the management of patients with ALF [39, 314, 446, 447] and the earlier report that hypothermia led to a delay in the onset of coma and brain edema in hepatic devascularized rats [254], the effect of hypothermia on cytokine expression in the serum and brains of these animals was also examined.

Materials and methods

Surgical Procedures

Adult male Sprague-Dawley rats (200 to 250 g) purchased from Charles River (Saint-Constant, Quebec, Canada) were tested routinely for common pathogens and were free from infection at the time of surgery. Rats were anesthetized using isoflurane, and an end-to-side portacaval anastomosis was performed according to the guidelines of Lee and Fisher (1961)[448]. Briefly, rats underwent a laparotomy, the inferior vena cava and portal vein were isolated and clamped using an anastomosis clamp (Roboz Instruments Inc, Washington DC, USA), and an elliptical portion 1.5 times the diameter of the portal vein was removed. The portal vein was ligated and

cut, and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was < 30mins. Sham-operated control rats, matched for weight, were anesthetized similarly and the inferior vena cava was clamped for 20 mins. After surgery, all animals were individually housed with free access to food and water under constant conditions of temperature, humidity, and light cycles. Twenty-four hours after portacaval anastomosis, rats were reanesthetized and subjected to hepatic artery ligation (HAL). After HAL, arterial blood glucose levels were monitored and glucose was administered subcutaneously as needed to maintain normoglycemia. Body temperature was monitored every 15 min and maintained at 37°C ± 0.5°C using heating pads. Hypothermia occurred spontaneously in the absence of external heating, and body temperature in the hypothermic ALF animals was again maintained at 33°C±0.5°C using heating pads when necessary. A group of animals was killed at precoma (before the appearance of encephalopathy and brain edema) 6 h after HAL (ALF-6 h). A second group (ALF-coma) was killed at the coma stage of encephalopathy (defined as the loss of righting and corneal reflexes), at which time the animals had significant brain edema. Hypothermic animals (ALF-33) were killed in parallel with time-matched comatose normothermic ALF animals and sham-operated controls. Their brains were rapidly removed, dissected on ice, and were immediately frozen in isopentane. All tissues were stored at -70°C until use. All the above surgical methods were performed in accordance with the Guidelines of Canadian Council of Animal care and were approved by the Animal Research Committee at Saint-Luc Hospital (C.H.U.M.).

Brain Water Measurement

Brain water was quantitated using the wet-weight/dryweight method. Half of the brain sample was weighed before and after 48 h of incubation in a 120°C oven. Water content of the brain samples are expressed as percentage of water content according to the following equation: %water = (wet weight–dry weight)/wet weight × 100.

Ammonia Concentration

Plasma and CSF ammonia concentrations were determined using a commercial ammonia test kit (Sigma Aldrich, St-Louis, MO, USA) on the basis of the enzymatic method using the glutamate dehydrogenase reaction [449].

Immunohistochemistry

Animals were deeply anesthetized using pentobarbital (60 mg/kg). After being transcardially perfused with 240-mL ice-cold saline followed by 240mL of neutral-buffered formalin (containing 4% formaldehyde, 0.5% sodium phosphate buffer, 1.5% methanol, pH 7.0), their brains were removed, postfixed in 10% formalin at 4°C for 12 h, and transferred into an ice-cold phosphate-buffered saline (PBS) solution for storage. Coronal sections that were 50-µm thick were obtained using a vibratome from -4.0 to -5.5 mm relative to the bregma according to the rat brain atlas of Paxinos and Watson (1986) [450]. Sections were incubated with 0.3% hydrogen peroxide in

PBS for 10min to block the endogenous peroxidase activity. After washing with PBS, the sections were blocked with 2% horse serum and 0.5% Triton X-100 in PBS, washed, and incubated at 4°C overnight with mouse anti-CD11b/c (OX-42) (1/1000) (Cedarlane Laboratories, Burlington, NC, USA). After washing with PBS, the sections were incubated for 1 h with horse antimouse biotinylated secondary antibody (1:100) (Vector Laboratories, Burlingame, CA, USA). Furthermore, after washing with PBS, the sections were incubated with the Vectastain ABC reagent (Vector Laboratories) and immunoreactivity was detected by incubation with 3-3'-diaminobenzidine containing urea–hydrogen peroxide (Sigma Aldrich). The sections were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, CA, USA), dehydrated stepwise in ethanol and xylene, and coverslipped with Permount (Fisher Scientific). Sections without primary antibodies were used as negative controls and showed no immunoreactivity. Quantitative analysis was performed by counting the immunopositive cells in 10 selected representative areas of 0.5 mm^2. Cell counts were performed by an investigator who was unaware of the animal treatment group.

Measurement of Cytokine Gene Expression by Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

In view of the established diurnal variations of IL-1β and TNF-α mRNAs, all experiments were performed at the same time of day (pm). Animals were transcardially perfused with 240mL ice-cold saline to remove the residual blood from the brain. Total RNA was isolated from rat brain cortex using the Trizol reagent

(Invitrogen Ltd, Carlsbad, CA, USA), according to the manufacturer's instructions. cDNA was synthesized using thermoscript RT-PCR system (Invitrogen). Expression levels were assessed using real-time PCR in a RotorGene 3000^{TM} Real time DNA detection system (Corbett Life Science, Sydney, Australia) with the Quantitect SYBRGreen I PCR kit (Qiagen, Valencia, CA, USA). Oligonucleotide primers were designed using the Primer 3 software [451] on the basis of the following GeneBank accession numbers: V01217 (β-actin), M98820 (IL-1β), M26744 (IL-6), and X66539 (TNF-α), and included at least one intron. The specificity of the oligonucleotide primers was verified using the program BLASTN from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). For each primer pair, the amplified cDNA fragments were verified by agarose gel to confirm the absence of the intron and of any nonspecific products. The forward and reverse primers used were 5'-CCACAGCTGA GAGGGAAATC-3' and 5'-TCTCCAGGGAGGAAGAGGAT-3' for β-actin; 5'-TCTTCTCATTCCTGCTCGTG-3' and 5'-GATG AGAGGGAGCCCATTT-3' for TNF-α; 5'-CTCAACTGTGAAATAGCAGCTTTC-3' and 5'-GGACAGCCCAAGTCAAGG-3' for IL-1β; and 5'-CTTCACAAGTCGGAGGCTTAAT-3' and 5'-ACAGTGCATCATCGCTGTTC-3' for IL-6. A relative quantification was performed by comparing the threshold cycle values of samples with serially diluted standards. Expression levels were normalized to the housekeeping gene, β-actin.

Plasma, CSF Collection, and ELISA (Enzyme-Linked Immunosorbent Assay)

Plasma and CSF samples were collected 6 h after HAL (precoma) and at the coma stage of encephalopathy. Cisterna magna catheters were installed in groups of animals to collect CSF as described earlier [52]. In brief, the animal's head was mounted with the skull in a horizontal position on a stereotaxic apparatus. A 3-cm incision was made on the skin from the back of its head, and the overlying connective tissue was removed to expose the skull. A small hole was drilled in the skull using a dental burr (009) on the midline immediately rostral to the interparietal–occipital bone suture. The hole was drilled in such a way that the occipital bone could be used as a guideline while inserting the cannula (PE-10 tubing, Clay Adams, Parsipanny, NJ, USA). The catheter was inserted into the cisterna magna. Correctness of placement was accompanied by a spontaneous flow of clear CSF. Protein levels of IL-1β, IL-6, IL-10, and TNF-α were measured in plasma and in CSF samples using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA; Biosource, Camarillo, CA, USA) according to the manufacturer's instructions. The plates were read at 450 nm and the absorbances were converted to pg/mL using standard curves prepared with recombinant cytokines.

Statistical Analysis

All data are expressed as the mean ± s.e.m., and statistical analysis was performed using ANOVA (one-way analysis of variance) followed by Tukey's post hoc analysis. A probability of $P<0.05$ was chosen to establish significance between the groups. Data were analyzed using the Prism 4.0 software (Prism 4.0, San Diego, CA, USA).

Results

Neurologic Status and Brain Edema

After hepatic devascularization, normothermic ALF rats (maintained at 37°C) developed progressive encephalopathy, which progressed to loss of corneal reflex (coma). At 6 h after surgery (ALF-6 h), ALF rats showed no overt neurologic symptoms. Hypothermia (ALF rats maintained at 33°C) significantly delayed the onset of encephalopathy. Sham-operated controls and ALF-6 h rats showed normal behavior and unchanged reflexes throughout the entire period of the experiments.

Brain water content was not significantly different between sham-operated control rats and normothermic rats 6 h after HAL (79.4 ± 0.07% versus 79.5 ± 0.14%), but was elevated significantly in normothermic rats that were at the coma stage of encephalopathy (80.8 ± 0.06%, $P < 0.001$). Hypothermia (ALF-33) significantly attenuated brain water content (ALF-33 versus ALF-coma: 79.5 ± 0.15% versus 80.8 ± 0.06%, $P < 0.001$) in ALF rats compared with normothermic rats killed at equivalent time points (Figure 1).

Plasma and CSF Ammonia

Serum ammonia levels were elevated in normothermic ALF rats starting 6 h after HAL (ALF-6 h versus sham-operated controls: 729 ± 32 versus 62 ± 9 mmol/L, P<0.001) with further increases at the coma stage of encephalopathy (1566 ± 72 mmol/L, P< 0.001). CSF ammonia levels were also increased 6 h after HAL (ALF-6 h versus sham-operated controls: 1049 ± 78 versus 109 ± 7 mmol/L, P< 0.001) with further increases again apparent at the coma stage of encephalopathy (2067 ± 176 mmol/L, P<0.001). Hypothermia significantly attenuated the rise of both serum (ALF-33 versus ALF-coma: 637 ± 57 versus 1566 ± 72 mmol/L, P< 0.001) and CSF ammonia concentrations (ALF-33 versus ALF-coma: 682 ± 62 versus 2067 ± 176 mmol/L, P< 0.001) (Figure 1).

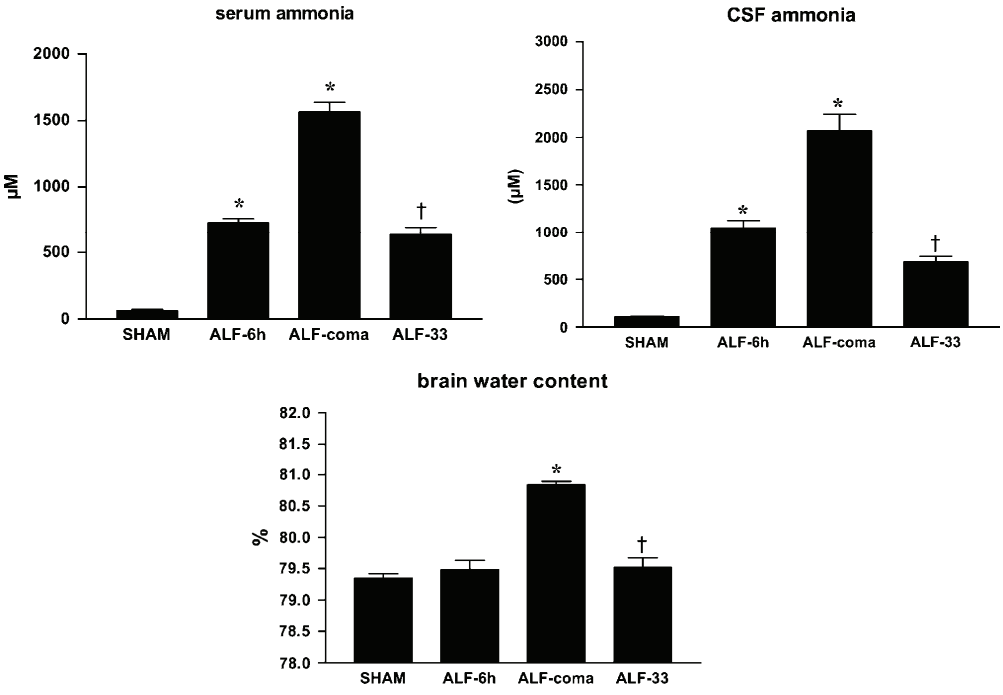

Figure 1: CSF and plasma ammonia and brain water content in ALF rats maintained normothermic (ALF-6 h, ALF-coma) or hypothermic (ALF-33) compared with sham-

operated controls (sham). Data represent mean±s.e.m. of 10 animals in each group. *P < 0.001 versus sham; †P < 0.001 versus ALF-coma.

Effects of ALF on Microglial Activation

Formaldehyde-fixed floating cerebral cortical sections from ALF rats stained with CD11b/c (OX-42), the major histocompatibility complex class II antigen marker, reveal microglial activation, which was correlated with the onset of brain edema and coma stage of encephalopathy (Figure 2). Comparable increases in OX-42-positive cells were also observed in the thalamus and hippocampus (Table 1).

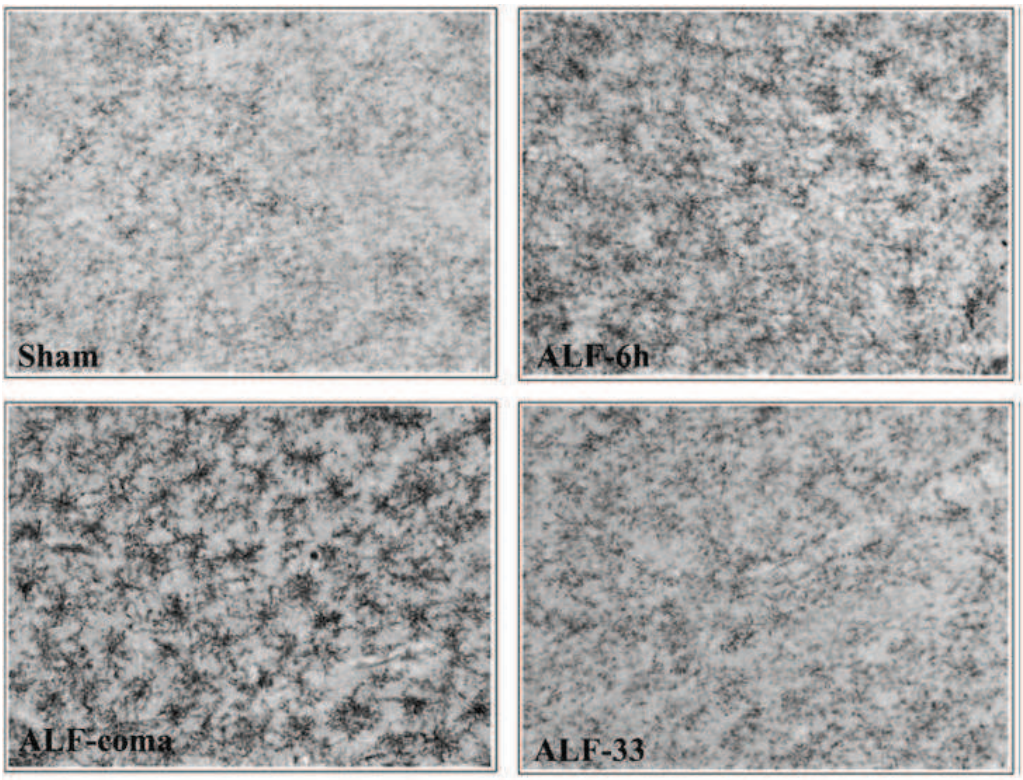

Figure 2: Microglial activation in the brains of rats with ALF at the coma stage of encephalopathy. Representative micrographs showing the effect of ALF on OX-42 staining in cerebral cortex from sham-operated controls (sham) and comatose ALF rats (ALF-coma) (original magnification 200 ×).

Table 1 Effect of hypothermia on microglial activation in various brain regions of ALF rats

	Frontal cortex	*Thalamus*	*Hippocampus*
Sham-operated controls	131.7 ± 4.0	107.9 ± 6.7	117.2 ± 3.8
ALF-6 h	201.0 ± 10.3*	155.4 ± 6.7*	169.4 ± 10.9*
ALF-coma	289.2 ± 7.2*	231.5 ± 8.8*	237.3 ± 6.5*
ALF-33	178.6 ± 12.6**	159.0 ± 7.2**	143.0 ± 4.4**

ALF, acute liver failure.
OX-42-positive cells (per x100 optical fields) in brain regions of sham-operated controls (sham), ALF-rats 6 h after HAL (ALF-6 h), ALF rats at coma stage of encephalopathy (ALF-coma), and in hypothermic ALF rats (ALF-33). Data represent mean ± s.e.m. of 10 measurements per animal ($n = 6$); (*$P < 0.001$ versus sham-operated controls; **$P < 0.001$ versus ALF-coma).

Effects of ALF and Hypothermia on Circulating IL-1β, IL-6, and TNF-α

In normothermic ALF rats, serum IL-1β protein levels were increased 3.8-fold at 6 h after HAL (sham-operated controls versus ALF-6 h: 36.7 ± 10.6 versus 140.2 ± 10.4 pg/mL, P< 0.05) and IL-6 levels were increased 40-fold (sham-operated controls versus ALF-6 h: 72.5 ± 1.8 versus 2933 ± 196 pg/mL, P< 0.001), whereas TNF-α concentrations remained at the level of sham-operated controls (sham versus ALF-6 h: 3.7 ± 0.3 versus 3.4 ± 0.3 pg/mL) (Figure 3). At coma stage, IL-1β concentrations were increased 6.5-fold (239.3 ± 49.3 pg/mL, P<0.001), IL-6 levels were increased 47-fold (3419 ± 218.3 pg/mL, P<0.001), and TNF-α concentrations were increased 2.8-fold (4.2 ± 0.7 pg/mL) (P < 0.001). In hypothermic ALF rats, increases in serum levels of IL-1β, IL-6, and TNF-α were attenuated by 55% (P<0.01), 81% (P<0.001), and 60% (P<0.001), respectively, compared with normothermic ALF rats (Figure 3).

Concentrations of the antiinflammatory cytokine IL-10 were increased 16-fold ($P<0.001$) in the plasma of normothermic ALF animals, but this increase was only marginally influenced by hypothermia (data not shown).

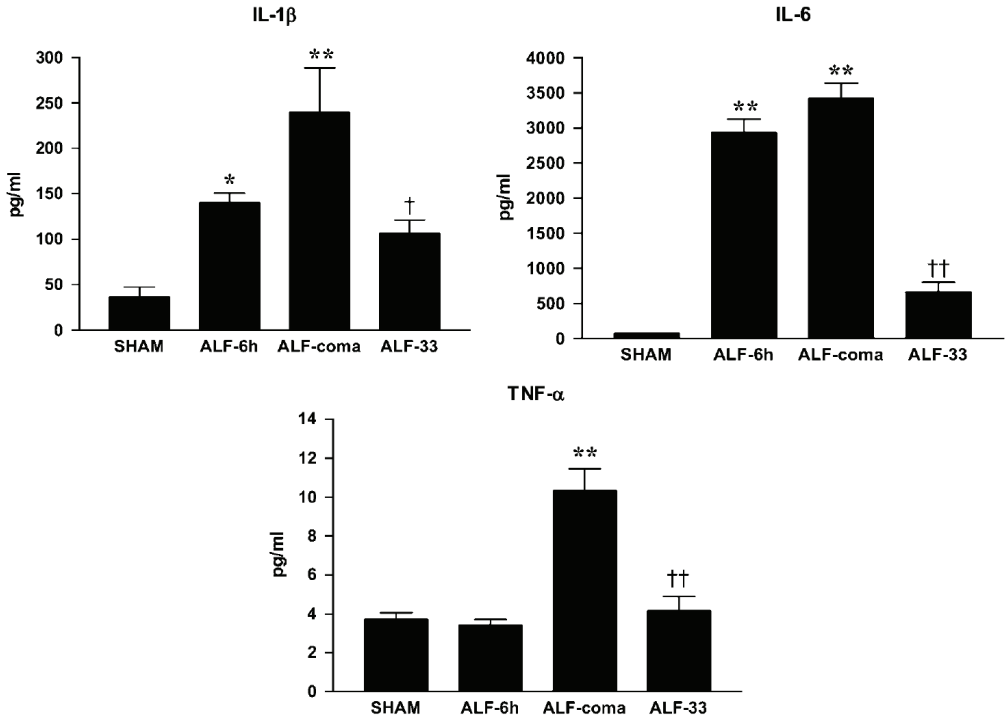

Figure 3: Plasma IL-1β, IL-6, and TNF-α levels in ALF rats maintained normothermic (ALF-6 h, ALF-coma) or hypothermic (ALF-33) compared with sham-operated controls (sham). Data represent mean ±s.e.m. of 10 animals in each group. *$P < 0.05$ versus sham; **$P < 0.001$ versus sham; $^{\dagger}P < 0.01$ versus ALF-coma; $^{\dagger\dagger}P < 0.001$ versus ALF-coma.

Effects of ALF and Hypothermia on Cerebral (CSF) IL-1β, IL-6, and TNF-α

The IL-1β protein levels in CSF were increased 2.3-fold in normothermic ALF rats at the coma stage of encephalopathy (ALF-coma versus shamoperated controls: 5.4 ± 0.6 versus 2.4 ± 0.3 pg/mL, $P<0.001$), but were not changed significantly in the 6 h(precoma) after-HAL group of animals (Figure 4). IL-6 protein concentrations were increased in normothermic ALF rats 6 h after HAL (shamoperated controls versus ALF-6 h: 35.8 ± 4.0 versus 60.7 ± 4.8 pg/mL, $P<0.05$) with further increases at the coma stage of encephalopathy (108.2 ± 9.5 pg/mL, $P<0.001$). The TNF-α levels in CSF were not changed significantly in the ALF-6 h group, but were increased significantly at the coma stage of encephalopathy (sham-operated controls versus ALF-coma: 1.1 ± 0.2 versus 2.3 ± 0.2 pg/mL, $P<0.001$). In hypothermic ALF rats, increases in CSF levels of IL-1β, IL-6, and TNF-α were attenuated by 65% ($P<0.001$), 67% ($P<0.001$), and 34% ($P<0.05$), respectively, compared with normothermic ALF rats (Figure 4). The antiinflammatory cytokine, IL-10, was increased fivefold ($P<0.001$) in the CSF of normothermic ALF animals, but again this increase was marginally influenced ($P>0.05$) by hypothermia (data not shown).

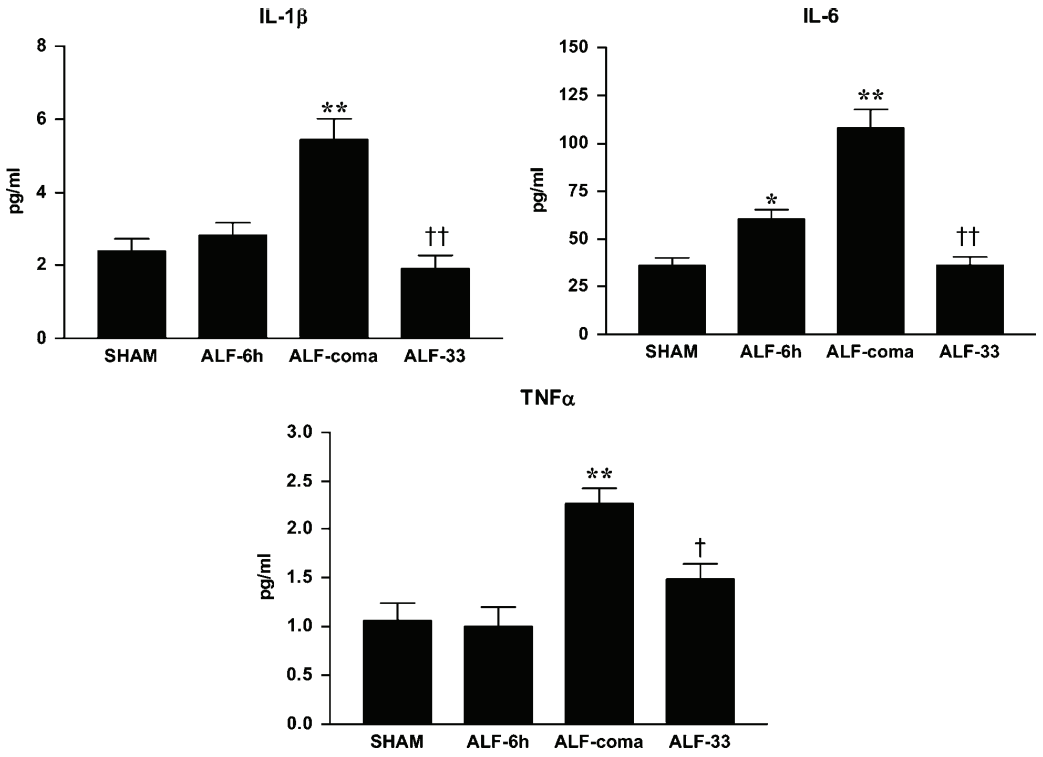

Figure 4: CSF IL-1β IL-6, and TNF-α levels in ALF rats maintained normothermic (ALF-6 h, ALF-coma) or hypothermic (ALF-33) compared with sham-operated controls (sham). Data represent mean ±s.e.m. of 10 animals in each group. *$P < 0.05$ versus sham; **$P < 0.001$ versus sham; $^{\dagger}P < 0.01$ versus ALF-coma; $^{\dagger\dagger}P < 0.001$ versus ALF-coma.

Effects of Hypothermia on IL-1β, IL-6, and TNF-α Gene Expression in ALF Rats

Brain samples from ALF rats were thoroughly perfused transcardially with saline to prevent the influence of circulating cytokines on measurements of brain cytokines. Using quantitative real-time PCR, cytokine mRNA levels were normalized to β-actin mRNA levels. The IL-1β gene expression in the brain was elevated 3.2-fold in

normothermic comatose ALF rats (ALF-coma versus sham-operated controls: 324.5 ± 41.3 versus 100 ± 10.2, P<0.001), but was not changed significantly 6 h after HAL (Figure 5). Brain IL-6 mRNA levels were increased 2.1-fold in normothermic ALF rats 6 h after HAL (sham-operated controls versus ALF-6 h: 100 ± 13.6 versus 208.6 ± 14.0, P<0.05) with further increases at the coma stage of encephalopathy (388.0 ± 42.0, P<0.001). TNF-α gene expression in the brain was increased 2.8-fold in normothermic ALF rats at the coma stage (sham-operated controls versus ALF-coma: 100.1 ± 8.8 versus 282.1 ± 26.5, P<0.001) but was not changed significantly 6 h after HAL. In hypothermic ALF rats, increases in brain IL-1β, IL-6, and TNF-α mRNA levels were attenuated by 89%, 47%, and 37%, respectively (P<0.001), compared with that in normothermic ALF rats (Figure 5).

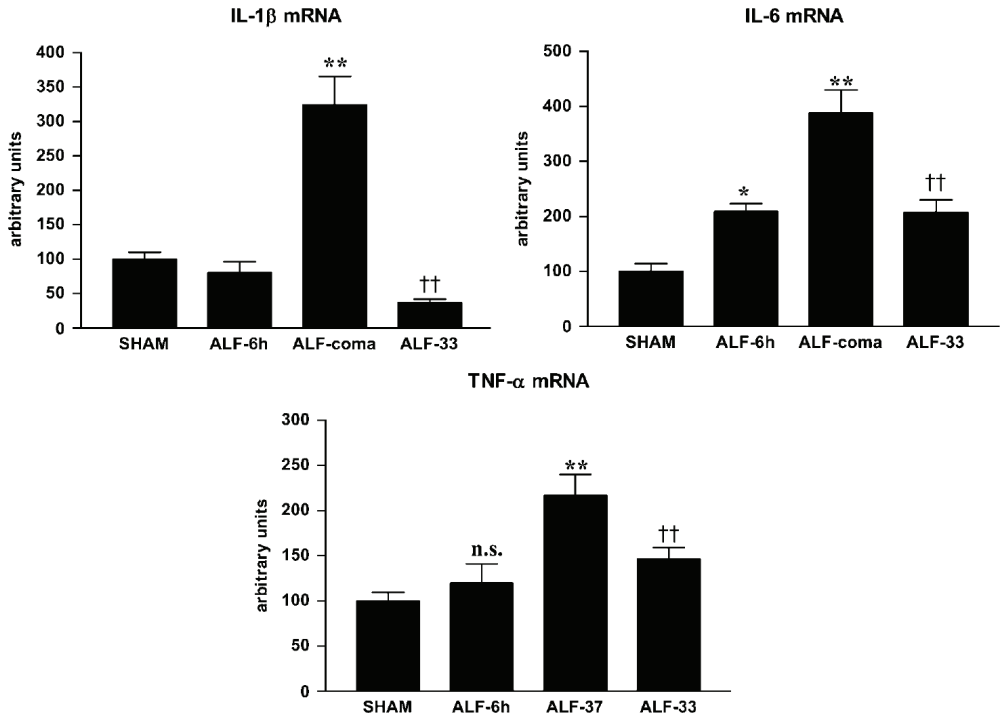

Figure 5: Brain IL-1β IL-6, and TNF-α mRNA levels in ALF rats maintained normothermic (ALF-6 h, ALF-coma) or hypothermic (ALF-33) compared with sham-operated controls (sham). Data represent mean±s.e.m.of 10 animals in each group. *P<0.05 versus sham; **P<0.001 versus sham; †P<0.05 versus ALF-coma; ††P<0.001 versus ALF-coma; NS: not significantly different from sham.

Discussion

Results of this study provide direct evidence for brain-derived proinflammatory mechanisms in the pathogenesis of the neurologic complications of ALF. This evidence includes activation of microglia, as shown by increased immunoreactivity of the major histocompatibility complex marker, OX-42, together with the increased production of brain proinflammatory cytokines. Increased cytokine transcripts in the hepatic devascularized rat model of ALF were found to be selective in terms of their identity, the magnitude of the increase in their expression, and the timing of the increased expression in relation to the progression of ALF. Early increases in IL-6 mRNA levels were followed by more substantial increases in the expression of IL-1β and TNF-α and occurrence of severe encephalopathy and brain edema was associated with a generalized increase in the expression of all three cytokines. Although astrocytes when activated, are theoretically an alternative source of cytokines, there is no evidence for reactive astrocytosis in this model of ALF [208]. The finding of increased brain proinflammatory cytokines in association with encephalopathy in ALF adds to a growing body of evidence implicating these cytokines in

neuropsychiatric disorders. Earlier studies show that expression of TNF-α in the brain results in poor performance in cognitive tasks [452], and that altered expression of TNF-α and IL-1 in the brain leads to impaired sleep quality [453]; in this regard, it is interesting to note that HE is characterized in its early stages by sleep disorders and cognitive deficits.

It may be tempting to speculate that the increased brain cytokines in this study originated from infection. Alternatively, apparent increases in brain cytokines could have originated from blood contamination of the brain tissue. However, neither possibility is likely, because the presence of infection was precluded by rigorous screening for bacteria and other pathogens in all animals before entry into the study. Blood contamination of the brain tissue was prevented by transcardial perfusion of all animals to remove residual blood from the brain before cytokine assays. Serum concentrations of TNF-α, IL-1β and IL-6 were increased significantly in hepatic devascularized rats (this study), and this cytokine profile resembles that reported earlier in rodent models of ALF resulting from acetaminophen [454] or azoxymethane [455] hepatotoxicity, as well as in sera from patients with ALF because of acetaminophen toxicity or viral hepatitis [299]. In this study, increases in IL-1β IL-6, and TNF-α levels were measured as a function of severity of liver failure and were sensitive to mild hypothermia. Given the similar identity of the increased serum and brain cytokines in this study, it may be tempting to conclude that increased brain cytokines originated from failing liver. There are known routes whereby peripheral cytokines can directly cross the blood–brain barrier [456].

However, a uniquely peripheral source of cytokines is unlikely, given the present findings of increased expression of genes coding for TNF-α, IL-1β and IL-6 together with the increased expression of their respective proteins in the brain.

The precise mechanism(s) that are responsible for microglial activation in ALF are unknown. However, one potential mechanism involves ammonia toxicity. ALF results in sustained hyperammonemia, and brain ammonia concentrations in the 1 to 5 mmol/L range have been reported [31]. Ammonia inhibits a-ketoglutarate dehydrogenase in rat brain mitochondria [97], resulting in decreased glucose oxidation and in lactate accumulation. Increased brain lactate production has been shown unequivocally in experimental ALF [115], and CSF lactate levels are positively correlated with the severity of HE in both experimental [161] and human [162] ALF. Results of this study show that increases in TNF-α, IL-6, and IL-1β levels in the brain follow a time course that is comparable with that of brain ammonia [31], and CSF [197] and brain [115] lactate suggestive of a pathophysiologic link. Moreover, cultured astroglia exposed to lactate in concentrations equivalent to those reported in brain in experimental ALF cause increased production and release of these cytokines [457]. Conversely, there is evidence to suggest that TNF-α IL-6, and IL-1β have the capacity to increase permeability of cerebrovascular endothelial cells to ammonia [458, 459], suggesting that their presence could increase the blood–brain barrier permeability to ammonia providing an explanation for the disproportionately increased brain ammonia reported in experimental ALF [31].

Mild hypothermia is increasingly being considered as a useful tool to prevent and treat intracranial hypertension in ALF [311, 445, 447]. Several mechanisms by which hypothermia exerts its beneficial effects have been proposed. Such mechanisms include improvement in hepatic function [446], improvement of brain energy metabolism [114, 124, 197], and effects on the expression of genes coding for oxidative stress proteins [460]. Results of this study suggest an additional mechanism, namely reduction of brain proinflammatory cytokines. Further studies are needed to determine whether mild hypothermia is beneficial when instigated after the onset of ALF. Approaches aimed at the reduction of brain levels of proinflammatory cytokines have the potential to limit the cerebral consequences of ALF.

Disclosure

The authors state no conflict of interest.

Article 2:

Cerebral inflammation contributes to encephalopathy and brain edema in acute liver failure: protective effect of minocycline

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Abstract

Encephalopathy and brain edema are serious complications of acute liver failure (ALF). The precise pathophysiologic mechanisms responsible have not been fully elucidated but it has been recently proposed that microglia-derived proinflammatory cytokines are involved. In the present study we evaluated the role of microglial activation and the protective effect of the anti-inflammatory drug minocycline in the pathogenesis of HE and brain edema in rats with ALF resulting from hepatic devascularisation. ALF rats were killed 6 h after hepatic artery ligation before the onset of neurological symptoms and at coma stages of encephalopathy along with their appropriate sham-operated controls and in parallel with minocycline-treated ALF rats. Increased OX-42 and OX-6 immunoreactivities confirming microglial activation were accompanied by increased expression of interleukins (IL-1β, IL-6) and tumor necrosis factor-alpha (TNF-α) in the frontal cortex at coma stage of encephalopathy in ALF rats compared with sham-operated controls. Minocycline treatment prevented both microglial activation as well as the up-regulation of IL-1β, IL-6 and TNF-α mRNA and protein expression with a concomitant attenuation of the progression of encephalopathy and brain edema. These results offer the first direct evidence for central proinflammatory mechanisms in the pathogenesis of brain edema and its complications in ALF and suggest that anti-inflammatory agents may be beneficial in these patients.

Keywords: acute liver failure, hepatic encephalopathy, microglia, minocycline, proinflammatory cytokines.

Introduction

ALF resulting from viral infection or toxic liver injury is a life-threatening condition. Mortality rates are extremely high with death resulting predominantly from brain herniation due to intracranial hypertension caused by brain edema. There is increasing evidence to suggest that inflammation plays a significant role in the pathogenesis of brain edema in ALF. For example, the presence of systemic inflammation is associated with a more rapid progression of encephalopathy and brain edema in ALF patients [303]. Systemic inflammation in ALF results in increased circulating levels of proinflammatory cytokines including tumor necrosis factor alpha (TNF-α), and the interleukins (IL-1β, IL-6) [294, 445]. Moreover, arterio-venous difference studies in ALF patients with uncontrolled intracranial hypertension suggest net brain efflux of TNF-α, IL-1β and IL-6 consistent with increased brain production of these cytokines [314, 461]. However, conclusive evidence for increased brain cytokine production in ALF is lacking.

The objective of the present study was to assess brain inflammatory mechanisms in ALF. In particular, microglial activation and expression of proinflammatory cytokines weremeasured as a function of encephalopathy and brain edema severity using a well-characterized animal model of ALF, the hepatic devascularized rat [462]. In a second series of studies, the beneficial effects of minocycline, a

semisynthetic tetracycline shown to limit microglial activation in a wide range of neurodegenerative conditions [463], was investigated. Effects of minocycline on progression of encephalopathy and brain edema in hepatic devascularized rats was studied as a function of microglial activation and expression of proinflammatory cytokines. Microglial activation was measured by western blotting (OX-6) and CD11b/c (OX-42) immunohistochemistry.

Materials and methods

Hepatic devascularization and treatments

Adult male Sprague-Dawley rats (200–250 g) purchased from Charles River (Saint-Constant, Quebec, Canada) were routinely tested for common pathogens and were free of infection at the onset of surgery. Animals were anesthetized with isoflurane, and an endto-side portacaval anastomosis was performed according to the guidelines of Lee and Fisher (1961)[448]. Briefly, rats underwent a laparotomy, the inferior vena cava and portal vein were isolated and clamped using an anastomosis clamp (Roboz Instruments Inc, Washington, DC, USA) and an elliptical portion 1.5 times the diameter of the portal vein was removed. The portal vein was ligated and cut, and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was < 30 min. Sham-operated control rats (n = 10), matched for weight, were similarly anesthetized and the inferior vena cava was clamped for 20 min. Following surgery, all animals were individually housed with free access to

food and water under constant conditions of temperature, humidity, and light cycles. Twenty four hours after portacaval anastomosis, rats were reanesthetized and subjected to hepatic artery ligation (HAL). Following HAL, arterial blood glucose levels were monitored and glucose was administered subcutaneously as needed to maintain normoglycemia. Body temperature was monitored every 15 min and maintained at 37^{o}C ± 0.5^{o}C by means of heating pads. A group of animals (n = 10) was killed 6 h following HAL (ALF-6 h) before the appearance of encephalopathy and the onset of brain edema and a second group (n = 10) was killed at coma stage of encephalopathy (ALF-coma) (defined as the loss of corneal reflexes). In a second series of experiments, group (n = 10) of ALF animals (ALF-mino) or sham-operated controls were administered minocycline (22.5 mg/kg twice daily; i.p.) on days -2, -1 and day 0 of HAL operation. Control rats (n = 10) were given equivalent volumes of saline. Minocycline-treated animals were killed in parallel with comatose saline-treated ALF controls. Brains were rapidly removed, dissected on ice and were immediately frozen in isopentane. All tissues were stored at –70^{o}C until use. In a third series of experiments (Table 1), a group (n = 10) of ALF animals was administered saline or minocycline and progression of encephalopathy was monitored until the appearance of precoma (loss of righting reflexes) or coma (loss of corneal reflexes) stages of encephalopathy. All the above procedures were conducted in accordance with the Guidelines of Canadian Council of Animal care and were approved by Animal Research Committee at Saint-Luc Hospital (C.H.U.M.).

Brain water measurement

Brain water was quantitated by the wet-weight/dry-weight method. Half of the brain was weighed before and after 48 h incubation in a 120^{o}C oven. Water content of the brain samples is expressed as percentage of water content according to the following equation: %Water = (Wet Weight - Dry Weight)/Wet Weight × 100.

Real-time reverse-transcription polymerase chain reaction (QRT-PCR)

Animals were transcardially perfused with 240 mL ice-cold saline to remove residual blood in the brain. Total RNA was isolated from rat brain cortex using the Trizol reagent (Invitrogen Ltd, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA was synthesized using thermoscript RT-PCR system (Invitrogen). Expression levels were assessed by real-time PCR in a RotorGene 3000^{TM} Real-time DNA detection system (Corbett Life Science, Sydney, Australia) with the Quantitect SYBRGreen I PCR kit (Quiagen, Valencia, CA, USA). Oligonucleotide primers (Invitrogen) were designed using the PRIMER3 program [451] at http://primer3.sourceforge.net/ and selected to include at least one intron. The specificity of the oligonucleotide primers was verified using the program BLASTN from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). For each primer pair, the amplified cDNA fragments were analyzed by agarose gel to confirm the absence of the intron and of any nonspecific PCR products. The forward and reverse oligonucleotide primer sequences were 5'-CCACAGCTGAGAGGGAAATC-3' and 5'-TCTCCAGGGAGGAAGAGGAT-3'

for β-actin (GeneBank accession #V01217); 5'-CTCAACTGTGAAATAGCAGCTTTC-3' and 5'-GGACAGCCCAAGTCAAGG-3' for IL-1β (GenBank accession #M98820); 5'-CTTCACAAGTCGGAGGCTTAAT-3' and 5'-ACAGTGCATCATCGCTGTTC-3' for IL-6 (GenBank accession #M26744); 5'-TCTTCTCATTCCTGCTCGTG-3' and 5'-GATGAGAGGGAGCCCATTT-3' for TNF-α (GenBank accession #X66539). A relative quantification was performed by comparing the threshold cycle values of samples with serially diluted standards. Expression levels were normalized to the housekeeping gene β-actin.

CSF removal and enzyme-linked immunosorbent assay

CSF samples were collected 6 h post-HAL and at the coma stage of encephalopathy. Cisterna magna catheters were installed in groups of animals just before the collection of CSF as previously described [52]. In brief, the animal's head was mounted with the skull in a horizontal position in a stereotaxic apparatus. A 3-cm incision was made in the skin from the back of the head and the overlying connective tissue was removed to expose the skull. A small hole was drilled in the skull using a dental burr (009) on the midline immediately rostral to the interparietal–occipital bone suture. The hole was drilled in such a way that the occipital bone could be used as guideline while inserting the cannula (PE-10 tubing, Clay Adams, Parsipanny, NJ, USA). The catheter was inserted into the cisterna magna. Correctness of placement was accompanied by a spontaneous flow of clear CSF. IL-1β, IL-6 and TNF-α protein levels were measured in CSF samples using a

quantitative sandwich enzyme immunoassay technique with monoclonal antibodies specific for rat IL-1β, IL-6 and TNF-α (R&D Systems, Minneapolis, MN, USA; Biosource, Camarillo, CA, USA). Detection limits were IL-6 (14 pg/mL), IL-1β (1 pg/mL), TNF-α (0.5 pg/mL). The plates were read at 450 nm and the absorbances were converted to pg/ml using standard curves prepared with recombinant cytokines.

Western blot analysis

Brain samples from frontal cortex were homogenized in ice-cold RIPA buffer [50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% Tergitol (Sigma-Aldrich, St-Louis, MO, USA), 1% sodium deoxycholate, 0.1% SDS] containing a protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 12 000 g for 45 min. Protein concentrations were measured using a DC Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (100 µg) were solubilized in Laemmli buffer (50 mM Tris–HCl, pH6.8, 10% glycerol; 2% SDS, 10% dithiothreitol, 0.1% bromophenol blue) and boiled for 10 min. Proteins were resolved by 8% denaturing SDS-polyacrylamide gels and transferred overnight to polyvinylidene difluoride membranes (Bio-Rad Laboratories). The filters were blocked for 1 h in Tris-buffered saline containing 5% dry milk and 0.1% Tween 20, then incubated for 1 h with mouse anti-OX-6 (1/250) (AbD Serotec, Raleigh, NC, USA) or with mouse anti-beta-actin (1/50 000) (Sigma-Aldrich). The blots were subsequently probed with the goat anti-mouse horseradish peroxidase-conjugated antiserum (Perkin-Elmer, Waltham, MA, USA) diluted 1/10 000 in the same buffer. After extensive washing with Tris-buffered saline, the

peroxidase activity was detected by chemiluminescence using the ECL detection system (Amersham, Arlington Heights, IL, USA). Expression levels were quantitated using an MCID-M1 imaging system (Imaging Research, ON, Canada). Results are expressed as percentage of the housekeeping protein β-actin to normalize for loading variation.

Immunohistochemistry

Animals were deeply anesthetized with pentobarbital (60 mg/kg; i.p.). After being transcardially perfused with 240 mL ice-cold saline followed by 240 mL neutral-buffered formalin (containing 4% formaldehyde, 0.5% sodium phosphate buffer, pH 7.0), brains were removed, post-fixed in 10% formalin at 4^{o}C for 12 h and transferred into ice-cold phosphate-buffered saline (PBS) solution for storage. 50 µm thick coronal sections were obtained using a vibratome from -4.0 mm to -5.5 mm relative to the bregma according to the rat brain atlas of Paxinos and Watson (1986) [450]. Sections were incubated with 0.3% hydrogen peroxide in PBS for 10 min to block endogenous peroxidase activity. Sections were blocked with 2% horse serum and 0.5% Triton X-100 and incubated at 4^{o}C overnight with mouse anti-CD11b/c (OX-42) (1/1000) (Cedarlane Laboratories, Burlington, NC, USA). After washing with PBS, sections were incubated for 1 h with horse anti-mouse biotinylated secondary antibody (1/100) (Vector Laboratories, Burlingame, CA, USA) and thereafter with Vectasain ABC reagent (Vector Laboratories). OX-42 immunoreactivity was detected by incubation with 3-3$^{'}$-diaminobenzidine containing

urea-hydrogen peroxide (Sigma-Aldrich). The sections were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, CA, USA), dehydrated stepwise in ethanol and xylene and coverslip with Permount (Fisher Scientific). Sections without primary antibodies were used as negative controls and showed no immunoreactivity. Quantitative analysis was performed by counting cell numbers positively stained in 10 selected representative areas of 0.5 mm^2. Cell counts were made by an investigator who was unaware of the animal treatment group.

Statistical analysis

All data are expressed as the mean ± SEM and statistical analysis was performed using unpaired student t-test (two group comparisons) or one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis (multiple comparisons). A probability of $p < 0.05$ was chosen to establish significance between the groups. Data were analyzed by using Prism 4.0 software (Prism 4.0, San Diego, CA, USA).

Results

Hepatic devascularization induces encephalopathy and brain edema

Following HAL, rats developed a progressive encephalopathy, starting with lethargy (evident 6 h after HAL) progressing to loss of righting and corneal reflexes and coma (Table 1). Sham-operated rats exhibited normal behavior and neurological reflexes

throughout the period of the experiments. Brain water content was not significantly different between sham-operated rats and ALF rats 6 h after HAL (ALF-6 h vs. sham: 78.23 ± 0.14% vs. 78.09 ± 0.07%), but was significantly elevated in ALF rats at coma stage (ALF-coma vs. sham: 80.78 ± 0.10% vs. 78.09 ± 0.07%, $p < 0.001$) (Fig. 1a).

Table 1 Effect of minocycline on the progression of encephalopathy

Progression of encephalopathy	ALF-saline	ALF-mino
Time to loss of righting reflexes (h)	9.73 ± 0.23	13.09 ± 0.47*
Time to loss of corneal reflexes (h)	12.97 ± 0.36	15.84 ± 0.39*

Time to loss of righting reflexes and loss of corneal reflexes in ALF rats treated with minocycline (ALF-mino) compared with saline-treated ALF controls (ALF-saline) expressed in hours (h). Data represent mean ± SEM of n= 10 animals per treatment group; significant changes compared with ALF saline group indicated by *$p < 0.001$ (Student's t-test).

Microglial activation occurs at coma stage of encephalopathy and correlates with the onset of brain edema

Formaldehyde-fixed floating cerebral cortical sections of ALF rats stained with CD11b/c (OX-42) (Fig.2a) show activation of microglia at the coma stage of encephalopathy (ALF-COMA) compared with sham-operated controls (SHAM). Comparable increases in numbers of OX-42-positive cells was also observed in the thalamus and hippocampus (Fig. 2b and Table 2). Western blot analysis of the major histocompatibility complex class II

antigen OX-6 at various time points during the progression of ALF reveals that microglial activation is correlated with the onset of brain edema and severe (coma stage) encephalopathy (ALF-coma) (Fig. 1b and c).

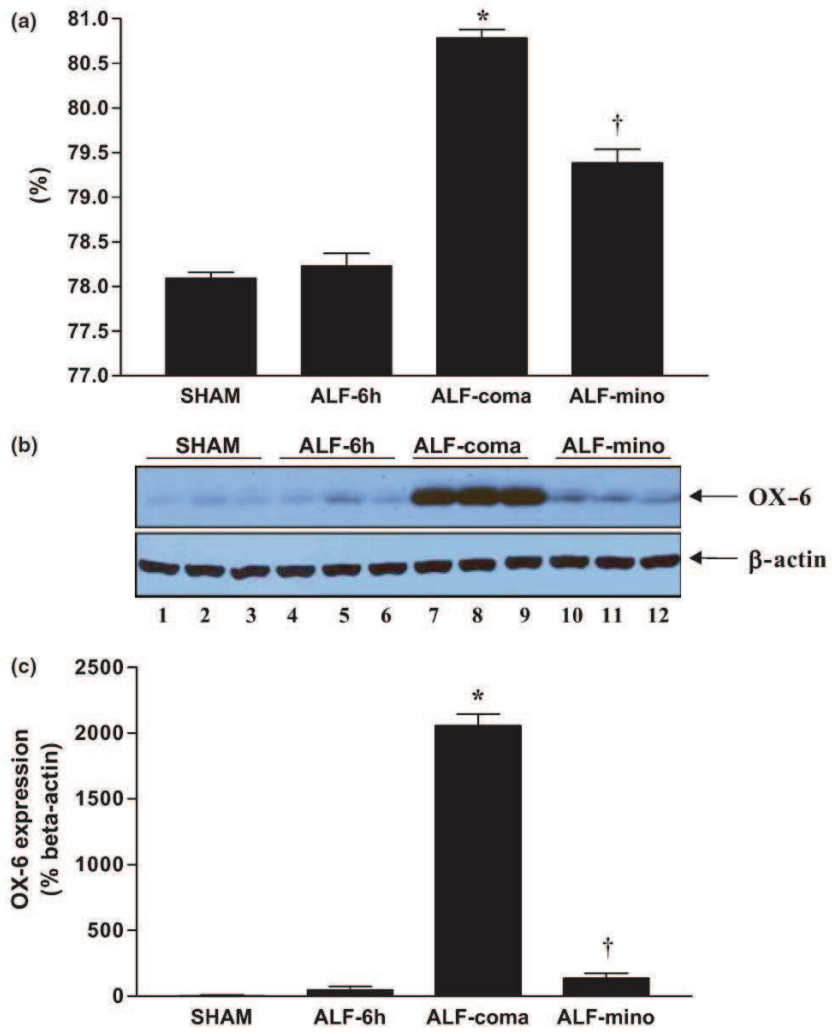


Fig. 1 Western blot analysis of OX-6 expression in ALF rats reveals that inhibition of microglial activation by minocycline treatment is correlated with attenuation of brain

edema. (a) Percentage of brain water content of cerebral cortex from sham-operated controls (sham), ALF rats 6h post-HAL (ALF-6 h), ALF rats at coma stage of encephalopathy (ALF-coma) and in ALF rats treated with minocycline (ALF-mino); (b) OX-6 protein expression in cerebral cortex from sham-operated controls (lanes 1–3), ALF rats 6 h post-HAL (lanes 4–6), ALF rats at coma stage of encephalopathy (lanes 7–9) and in ALF rats treated with minocycline (lanes 10–12); (c) Histogram representation of OX-6 expression in the various treatment groups. Data represent mean ± SEM of n= 10 animals per treatment group. Significant differences indicated by *$p < 0.001$ versus sham-operated controls and ALF-6 h; †$p < 0.001$ versus ALF-coma (ANOVA with post hoc Tukey's test).

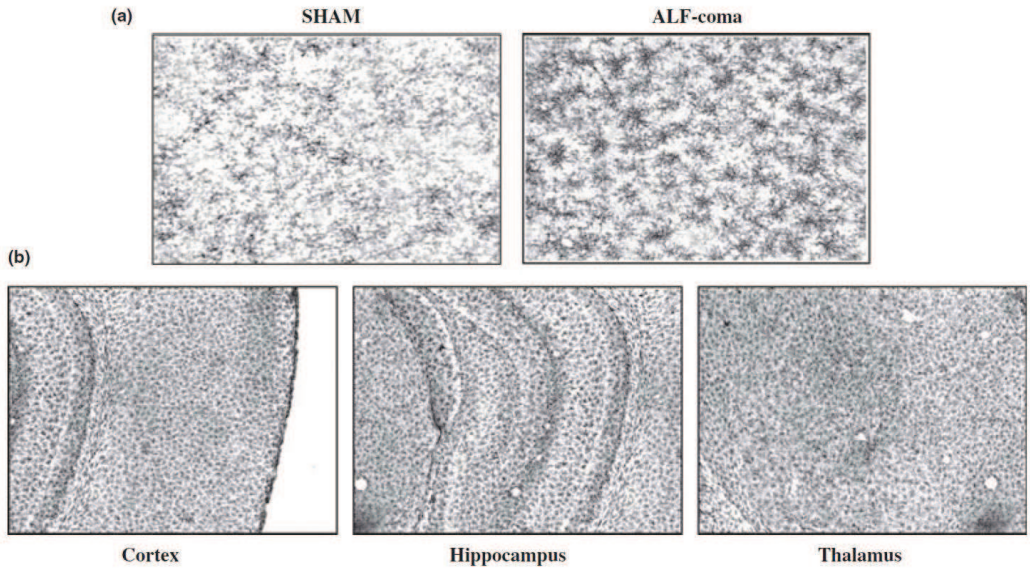


Fig. 2 Microglial activation in the brains of rats with ALF resulting from hepatic devascularization. Representative micrographs showing the effect of ALF on (a) CD11b/c (OX-42) immunoreactivity in cerebral cortex of sham-operated controls (SHAM) and ALF rats at coma stage of encephlopathy (ALF-COMA) (magnification: 200×) and (b) OX-42 staining in cerebral cortex, hippocampus and thalamus from ALF rats at coma stage of

encephalopathy (magnification: 50×). Note the generalized regional distribution of OX-42 immunostaining.

Table 2 Effect of minocycline on microglial activation

	Frontal cortex	Thalamus	Hippocampus
Sham-operated controls	28.5 ± 4.5	61.6 ± 4.0	41.0 ± 3.0
ALF-6 h	64.1 ± 3.7*	97.3 ± 5.7*	79.9 ± 4.0*
ALF-coma	147.3 ± 7.6*	163.1 ± 6.4*	138.8 ± 7.2*
ALF-mino	44.3 ± 2.8**	69.4 ± 4.4**	60.4 ± 2.2**

OX-42-positive cells (per × 200 optical fields) in brain regions of sham-operated controls, ALF-rats 6 h post-HAL (ALF-6 h), ALF rats at coma stage of encephalopathy (ALF-coma) and in ALF rats treated with minocycline (ALF-mino). Data represent mean ± SEM of 10 measurements per animal (n = 6); significant differences indicated by *$p < 0.001$ versus sham-operated controls and **$p < 0.001$ versus ALF-coma (ANOVA with post-hoc Tukey's test).

Minocycline treatment ameliorates the neurological status of ALF rats, prevents brain edema and attenuates microglial activation

In the saline-treated ALF group, rats progressively lost righting reflexes (precoma stage) 9.7 ± 0.2 h after HAL and lost corneal reflexes (coma stage) 13.0 ± 0.4 h after HAL. In comparison, minocycline-treatment significantly ($p < 0.001$) delayed the onset of

both precoma (loss of righting reflexes) and coma (loss of corneal reflexes) stages of encephalopathy to 13.1 ± 0.5 h and 15.8 ± 0.4 h after HAL, respectively (Table 1). Administration of similar doses of minocycline to sham-operated animal did not induce any neurological symptoms. As expected, minocycline was found to prevent microglial activation as shown by decreased OX-42 staining (Table 2) and OX-6 immunoreactivities (Fig. 1b and c). Analysis of brain water content in minocycline-treated ALF rats also revealed a significant attenuation of brain edema ($p < 0.001$) (Fig. 1a).

Minocycline treatment attenuates up-regulation of IL-1β, IL-6 and TNF-α expression in ALF rat brain

IL-1β, IL-6 and TNF-α protein levels in CSF were elevated 2.3-fold, 3.0-fold and 2.1-fold, respectively ($p < 0.001$) in comatose ALF rats (ALF-coma) compared with sham-operated control (sham) but were not significantly changed 6 h post-HAL (ALF-6 h) (Fig. 3). IL-1β, IL-6 and TNF-α gene expression in brain were elevated 3.2-fold, 3.9-fold and 2.8-fold, respectively ($p < 0.001$) in comatose ALF rats (ALF-coma) compared with sham-operated control (sham) but were not significantly changed 6 h post-HAL (ALF-6h) (Fig.4). Minocycline treatment prevented induction of IL-1β, IL-6 and TNF-α mRNAs and protein ($p < 0.001$) (Figs 3 and 4).

Minocycline treatment of sham-operated control animals had no significant effect on neurological status, brain water content or CSF cytokine levels (Fig S1).

Discussion

Results of the present study provide the first direct evidence for a role of cerebral inflammation in the pathogenesis of the encephalopathy and brain edema in ALF. Increased expression of the microglial marker proteins OX-6 and OX-42 in brain preparations indicative of microglial activation were found to predict the presence of severe encephalopathy (coma) and brain edema in rats with ALF due to hepatic devascularization. These findings add to a growing body of evidence that excessive microglial activation is a key feature of a wide range of neurological disorders that include neurodegenerative diseases as well as traumatic brain injury [356], stroke [357], and Wernicke's encephalopathy [358]. In some of these disorders, microglial activation occurs prior to the appearance of neuronal cell death. However, microglial activation may occur following treatment with subacute doses of lipopolysaccharide with no evidence of neuronal cell death [464]. Results of the present study represent a further such example; neuronal cell death is not a feature of ALF [465]. Microglial activation was accompanied by significant increases in expression of the proinflammatory cytokines IL-1β, IL-6 and TNF-α in brains of ALF rats at coma (edema) stages of encephalopathy. These findings constitute direct evidence for increased brain cytokines in experimental ALF and extend previous findings in ALF patients of arterio-venous differences for these cytokines [314, 461] consistent with their production in brain.

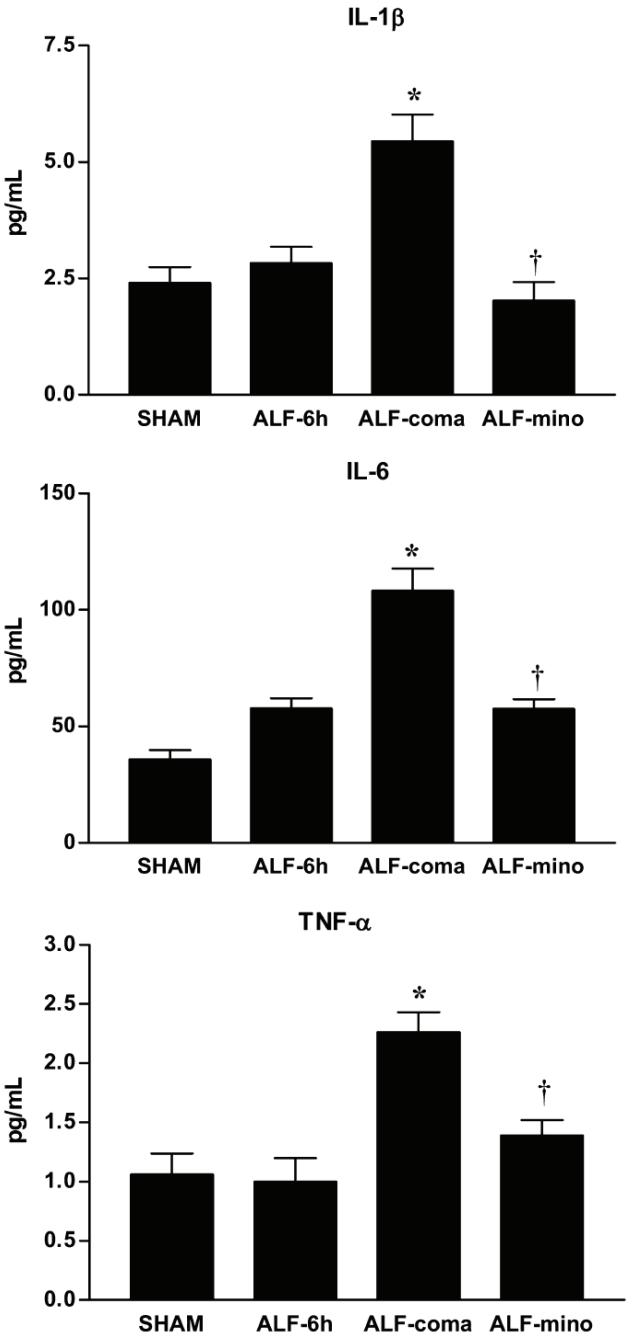


Fig. 3 Minocycline attenuates brain IL-1β, IL-6 and TNF-α expression in ALF rats. IL-1β, IL-6 and TNF-α protein levels were determined in the CSF of sham-operated controls

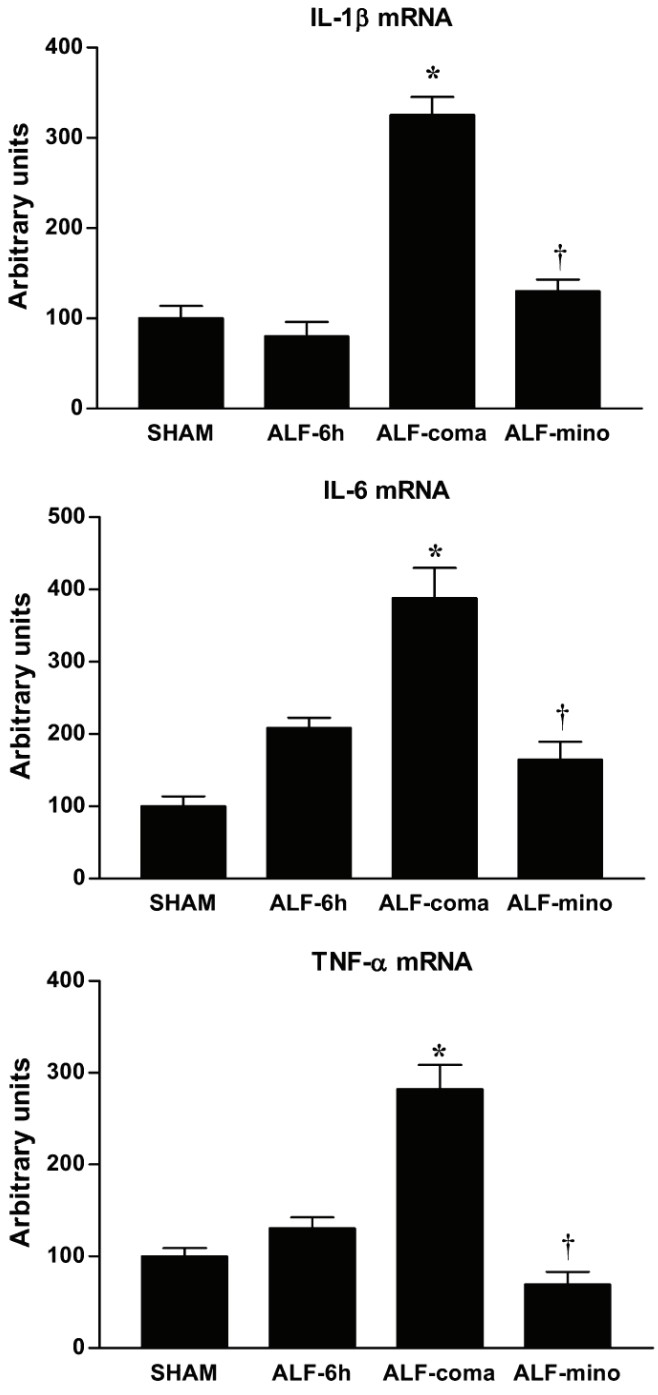
(sham), ALF rats 6 h post-HAL (ALF-6h), at coma stage of encephalopathy (ALF-coma) and ALF rats treated with minocycline (ALF-mino). Data represent mean ± SEM of n= 10 animals in each group. Significant differences indicated by *$p < 0.001$ versus sham-operated controls and ALF-6 h; †$p < 0.001$ versus ALF-coma (ANOVA with post hoc Tukey's test).

Hepatic encephalopathy in ALF is characterized by both cognitive and sleep disturbances, both of which could potentially result from increased brain cytokines. For example, over-expression of TNF-α and IL-1β in brain result in poor performance in cognitive tasks [452] and sleep dysregulation [466]. More importantly in the context of ALF, previous studies show that brain edema is significantly correlated with the presence of proinflammatory cytokines such as TNF-α and IL-1β [467, 468] and transient brain edema results from intracerebral administration of IL-1β to normal rats [469].

As expected from its anti-inflammatory properties [470-472], results of the present study demonstrate that treatment with minocycline reduces microglial activation and leads to significant decreases in expression of IL-1β, IL-6 and TNF-α in the brains of ALF rats. Moreover, these effects of minocycline were accompanied by significant decreases in brain water content, strengthening the notion that microglial activation and brain accumulation of inflammatory cytokines are implicated in the pathogenesis of brain edema in ALF. These findings are in line with previous reports that minocycline treatment is effective in limiting brain edema that accompanies

intracerebral hemorrhages [473]. Moreover, brain edema resulting from experimental hypoxia/ischemia is significantly reduced in IL-1 receptor-deficient animals [474]. However, results of the present study reveal that an almost complete normalization of IL-1β mRNA and protein following minocycline treatment led to significant but incomplete inhibition of brain edema in ALF animals suggesting that other cytokines or other pathophysiologic mechanisms could also be implicated.

Brain edema in ALF is cytotoxic in nature resulting almost exclusively from swelling of astrocytes [17, 206]. Moreover, experimental ALF results in significant alterations in expression of genes coding for key astrocytic proteins with the ability to modulate astrocyte volume including a loss of glial fibrillary acidic protein [208], the glutamate transporter EAAT-2 [262], the glucose transporter GLUT-1 [193], aquaporin IV [475] and the mitochondrial peripheral-type benzodiazepine receptor [217]. Although such protein modifications had previously been attributed to the toxic actions of ammonia in ALF, there is evidence to suggest that proinflammatory cytokines could also be implicated. For example, intracerebral administration of IL-1β leads to up-regulation of peripheral-type benzodiazepine receptor sites [476] while exposure of cultured astrocytes to IL-1β and TNF-α up-regulate aquaporin IV [477] and down-regulate glial fibrillary acidic protein [478] respectively. Moreover, high affinity transport of glutamate into cultured astrocytes is significantly impaired by exposure to IL-1β consistent with down-regulation of glutamate transporters [258, 479]. Whether or not treatment with minocycline would be beneficial in preventing these changes in protein expression in ALF awaits further studies.


IL-1β mRNA
Arbitrary units
400
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ALF-6h
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ALF-mino
TNF-α mRNA
Arbitrary units
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ALF-coma
ALF-mino

Fig.4 Minocycline attenuates brain IL-1β, IL-6 and TNF-α gene expression in ALF rats. Cytokine mRNAs were determined in cerebral cortex of sham-operated controls (sham), ALF rat 6 h post-HAL (ALF-6h), at coma stage of encephalopathy (ALF-coma) and ALF rats treated with minocycline (ALF-mino). Data represent mean ± SEM of n= 10 animals in each group. Significant differences indicated by *$p < 0.001$ versus sham-operated controls and ALF-6 h; †$p < 0.001$ versus ALF-coma (ANOVA with post hoc Tukey's test).

Findings of increased brain cytokines is generally considered to reflect increases in their local synthesis by microglia or cerebrovascular endothelial cells. However, some cytokines may be transported through circumventricular organs that lack a blood–brain barrier [480]. Furthermore, vagal or sympathetic afferent cytokine signaling has also been proposed although the importance of this latter route has been questioned [456]. Circulating levels of TNF-α and IL-1β are significantly increased in ALF [306] and, although neuropathologic investigations reveal no significant alterations of the blood–brain barrier in ALF [17], entry of cytokines via circumventricular organs remains a possibility. Alternative (or additional) mechanisms could involve brain accumulation of ammonia and lactate in ALF. Brain concentration of ammonia [31] and lactate are correlated with severity of encephalopathy [115, 161] and EEG changes [481] in experimental ALF and pathophysiologically-relevant concentrations of ammonia [482] and lactate [457] have been shown to cause release of TNF-α and IL-1β from cultured microglial cells.

The findings of microglial activation and increased brain proinflammatory cytokines may have important implications for current and future therapies in ALF. Albumin dialysis improves neurological status in ALF patients and removes circulating cytokines [483] and mild hypothermia shown to reduce ICP in ALF patients [39] was recently shown to lower brain concentrations of TNF-α and IL-1β in experimental ALF [484]. Minocycline has been shown to be effective in the treatment of multiple sclerosis in clinical trials [485]. However, long-term use of minocycline may result in liver damage [486], a property that could limit its use in ALF. Experimental therapeutic studies with other anti-inflammatory agents with the capacity to limit microglial activation and brain production of proinflammatory cytokines in ALF are warranted.

Acknowledgments

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Supporting information

Additional Supporting Information may be found in the online version of this article: Figure S1 Effects of minocycline on brain water content and proinflammatory cytokines in CSF of sham-operated controls. Please note: Wiley-Blackwell are not

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Article 3:

Hypothermia attenuates oxidative / nitrosative stress, encephalopathy and brain edema in acute (ischemic) liver failure

Neurochemistry International 55 (2009) 124–128

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ABSTRACT

Encephalopathy and brain edema are serious complications of acute liver failure (ALF). The precise pathophysiologic mechanisms responsible have not been fully elucidated but it has been suggested that oxidative/nitrosative stress is involved. In the present study we evaluated the role of oxidative/ nitrosative stress in the pathogenesis of HE and brain edema in rats with ALF resulting from hepatic devascularization. We also studied the effect of hypothermia, a treatment previously shown to delay the progression of encephalopathy and the onset of brain edema, on ALF-induced oxidative stress. ALF rats were sacrificed at precoma and coma stages of encephalopathy along with their appropriate sham-operated controls. Hypothermic ALF rats were sacrificed in parallel with normothermic comatose ALF rats. Nitric oxide production in plasma and brain was assessed indirectly by measuring the level of its stable end products, nitrite/nitrate (NOx), using the Griess reagent. Expression of nitric oxide synthase (NOS) isoforms and heme oxygenase-1 (HO-1) were measured using real-time quantitative PCR, Western blot analysis and immunohistochemistry. Increased nitrite/nitrate levels were observed in the plasma and frontal cortex in ALF rats at coma stage of encephalopathy compared to sham-operated controls. Increased expression of HO-1 protein and mRNA was observed in the frontal cortex of ALF rats at both precoma and coma stages of encephalopathy. Significant increases in expression of endothelial and inducible NOS mRNA isoforms also occurred at precoma and coma stages of encephalopathy. Expression of the neuronal nitric oxide synthase isoform

(nNOS) was not altered by ALF. Hypothermia normalized nitrite/nitrate levels in brain and significantly attenuated HO-1, eNOS and iNOS expression. These results suggest that, oxidative/nitrosative stress participates in the pathogenesis of brain edema and its complications in ALF and that the beneficial effect of hypothermia depends in part on its ability to inhibit oxidative/nitrosative stress-related mechanisms.

Keywords: Hepatic encephalopathy, Acute liver failure, Nitric oxide synthase, Oxidative/nitrosative stress, Brain edema, Hypothermia

1. Introduction

ALF resulting from viral infection or toxic liver injury is a life-threatening condition. Mortality rates are extremely high with death resulting predominantly from brain herniation due to intracranial hypertension caused by cytotoxic brain edema. Hyperammonemia is a critical factor in the pathogenesis of cerebral edema and herniation development following ALF [79]. While the precise mechanism responsible for ammonia's toxic effects on CNS function are unclear, there is increasing evidence to suggest that oxidative/ nitrosative stress plays a significant role. ROS are produced in rat brain following acute ammonia intoxication [487] as well as in cultured astrocytes exposed to pathophysiologic concentrations of ammonia [99]. Acute ammonia intoxication also leads to glutathione depletion,

lipid peroxidation and decreased activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase [488].

The objective of the present study was to assess oxidative stress in relation to encephalopathy and brain edema in ALF. Nitrite/nitrate levels as well as expression of heme oxygenase-1 (HO-1) and the NOS isoforms were measured as a function of encephalopathy and brain edema severity using a wellcharacterized animal model of ALF, the hepatic devascularized rat [462]. In a second series of studies, the beneficial effects of mild hypothermia previously shown to delay the progression of encephalopathy and the onset of brain edema in experimental ALF [254] as well as in ALF patients [489] was evaluated in relation to ALF-mediated oxidative stress markers and progression of neurological symptoms.

2. Materials and methods

2.1. Hepatic devascularization and treatments

Adult male Sprague-Dawley rats (200–250g) purchased from Charles River (Saint-Constant, Quebec, Canada) were routinely tested for common pathogens and were free of infection at the onset of surgery. Animals were anesthetized with isoflurane, and an end-to-side portacaval anastomosis was performed according to the guidelines of Lee and Fisher (1961)[448]. Briefly, rats underwent a laparotomy, the inferior vena cava and portal vein were isolated and clamped using an

anastomosis clamp (Roboz Instruments Inc., Washington DC) and an elliptical portion 1.5 times the diameter of the portal vein was removed. The portal vein was ligated and cut, and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was <30 min. Sham-operated control rats (n = 10), matched for weight, were similarly anesthetized and the inferior vena cava was clamped for 20 min. Following surgery, all animals were individually housed with free access to food and water under constant conditions of temperature, humidity, and light cycles. Twenty-four hours after portacaval anastomosis, rats were reanesthetized and subjected to hepatic artery ligation (HAL). Following HAL, arterial blood glucose levels were monitored and glucose was administered subcutaneously as needed to maintain normoglycemia. Body temperature was monitored every 15 min and maintained at 37 ± 0.5 °C by means of heating pads. Hypothermia occurred spontaneously in the absence of external heating and body temperature was maintained at 33 ± 0.5 °C using heating pads when necessary. A group of animals (n = 10) was sacrificed 6 h following HAL (ALF-6h) and a second group (n =10) was sacrificed at coma stage of encephalopathy (ALF-coma) (defined as the loss of corneal reflexes occurring 14–18 h post-HAL). Hypothermic animals (ALF-33) were sacrificed in parallel with time-matched comatose normothermic ALF animals and sham-operated controls. Brains were rapidly removed, dissected on ice and were immediately frozen in isopentane. All tissues were stored at -70 °C until use. All the above surgical methods were conducted in accordance with the Guidelines of Canadian Council of Animal care and were approved by Animal Research Committee at Saint-Luc Hospital (C.H.U.M.).

2.2. Brain water measurement

Brain water was quantitated by the wet-weight/dry-weight method. Half of the brain was weighed before and after 48 h incubation in a 120 °C oven. Water content of the brain samples is expressed as percentage of water content according to the following equation: % water = (wet weight - dry weight)/wet weight × 100.

2.3. Ammonia measurements

Immediately after decapitation, the head was flash frozen in isopentane cooled to liquid nitrogen temperature and the brain was subsequently chiselled out with special care to keep it frozen at all times. Brains were stored at -70 °C until analysis at which time frozen brains were weighed and powdered over liquid nitrogen. Frozen powder was extracted by homogenization in ice-cold 1 M perchloric acid and centrifuged at 16,000 × g for 15 min at 4°C. 50 µL of supernatant were added to a suspension of Dowex 50WX8-200 ion exchange resin (Sigma–Aldrich, St.-Louis, MO) and allowed to react for 5 min with constant shaking. After extensive washing, the colorimetric reaction was initiated by the addition of phenol and hypochlorite in the presence of nitroferricyanide. Absorbance was measured using a spectro-photometer at a wavelength of 630 nm. Plasma ammonia concentrations were determined using a commercial ammonia test kit (Sigma–Aldrich) based on the enzymatic method using the glutamate dehydrogenase reaction.

2.4. Nitrite/nitrate levels (NOx)

Nitrite concentrations in brain and plasma were determined using Griess reagent [490]. Brain tissue were homogenized in water and diluted to 5 µg/µl. Brain and plasma samples (100 µl) were diluted 2-fold in water and deproteinized by adding 10 µl of 30% ZnSO4. After centrifugation (10,000 × g, 5 min), the supernatant was incubated with cadmium for 24 h to reduce nitrate to nitrite. Samples (100 µl) or serial dilution of NaNO2 standard (linear range 0–100 µM) were applied to a microtiter plate well. Total nitrite in each sample was then determined by adding 50 µl Griess reagent 1 (1% sulfanilamide in 1 N HCl), followed by Griess reagent 2 (0.1% N-(1-napthyl)-ethylenediamine). The mixture was incubated for 10 min in the dark. Optical density was measured at 540 nm spectrophotometrically. Nitrate/nitrite levels were expressed as micromoles per gram tissue protein or micromoles per liter.

2.5. Western blot analysis

Brain samples from frontal cortex were homogenized in ice-cold RIPA buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Sigma–Aldrich) and centrifuged at 12,000 × g for 45 min. Protein concentrations were measured using a DC Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins (100 µg) were

solubilized in Laemmli buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol; 2% SDS, 10% dithiothreitol, 0.1% bromophenol blue) and boiled for 10 min. Proteins were resolved by 10% denaturing SDS-polyacrylamide gels and transferred overnight to PVDF membranes (Bio-Rad Laboratories). The filters were blocked for 1 h in Tris-buffered saline (TBS) containing 5% dry milk and 0.1% Tween 20, then incubated for 1 h with rabbit anti-HO-1 (1/700) (Assay Design, Ann Arbor, MI). The blots were subsequently probed with the goat anti-rabbit horseradish peroxidase-conjugated antiserum (PerkinElmer, Waltham, MA) diluted 1/10,000 in the same buffer. After extensive washing with TBS, the peroxidase activity was detected by chemiluminescence using the ECL detection system (Amersham, Arlington Heights, IL). Equal loading of proteins was confirmed by Ponceau S staining. Expression levels were quantitated using an MCID-M1 imaging system (Imaging Research, ON, Canada).

2.6. Real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Animals were transcardially perfused with 240 ml ice-cold saline to remove residual blood in the brain. Total RNA was isolated from rat brain cortex using the Trizol reagent (Invitrogen Ltd., Carlsbad, CA), according to the manufacturer's instructions. The cDNA was synthesized using thermoscript RT-PCR system (Invitrogen). Expression levels were assessed by real-time PCR in a RotorGene 3000^{TM} Real time DNA detection system (Corbett Life Science, Sydney, Australia) with the Quantitect SYBRGreen I PCR kit (Quiagen, Valencia, CA). Oligonucleotide

primers (Invitrogen) were designed using the PRIMER3 program [451] at http://primer3.sourceforge.net/ and selected to include at least one intron. The specificity of the oligonucleotide primers was verified using the program BLASTN from the National Center for Biotechnology Information (NCBI, Bethesda, MD). For each primer pair, the amplified cDNA fragments were analyzed by agarose gel to confirm the absence of the intron and of any nonspecific PCR products. The forward and reverse oligonucleotide primer sequences were 5'-CCACAGCTGAGAGGGAAATC-3' and 5'-TCTCCAGGGAGGAAGAGGAT-3' for β-actin (GeneBank accession # V01217); 5'-TCAACAGTGGGAGCAAAGC-3' and 5'-CTTTGT- GCGATTTGCCATC-3' for nNOS (GenBank accession # X59949); 5'-GATCCAGTGGGGGAAACTG-3' and 5'-GCCTCGGTTTGTTGCATACT-3' for eNOS (GenBank accession # AB176831); 5'-CCAAGGTGACCTGAAAGAGG-3' and 5'-TTGATGCTTGTGACTCTTAGGG-3' for iNOS (GenBank accession # U26686); 5'- GAGCCAGCCTGAACTAGCC-3' and 5'-GATGTGCACCTCCTTGGTG-3' for HO-1 (GenBank accession # J02722). A relative quantification was performed by comparing the threshold cycle values of samples with serially diluted standards. Expression levels were normalized to the housekeeping gene β-actin.

2.7. Statistical analysis

All data are expressed as the mean ± S.E.M. and statistical analysis was performed using unpaired Student's t-test (two-group comparisons) or one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis (multiple comparisons). A

probability of $p<0.05$ was chosen to establish significance between the groups. Data were analyzed by using Prism 4.0 software (Prism 4.0, San Diego, CA).

3. Results

3.1. Mild hypothermia improves neurological status and prevents brain edema in hepatic devascularized ALF rats

Following HAL, rats developed a progressive encephalopathy, starting with lethargy (evident 6 h after HAL) progressing to loss of righting and corneal reflexes and coma. Sham-operated control rats exhibited normal behavior and neurological reflexes throughout the period of the experiments. Brain water content was not significantly different between sham-operated rats and ALF rats 6 h after HAL (ALF-6 h) but was significantly elevated in ALF rats at coma stages (sham vs. ALF-coma: 78.04 ± 0.19% vs. 80.83 ± 0.05%, $p< 0.001$) (Table 1). Mild hypothermia significantly ($p < 0.001$) delayed progression of encephalopathy i.e. time to precoma (loss of righting reflexes) and time to coma (loss of corneal reflexes) (Table 2). Brain water content was also significantly reduced (78.31 ± 0.09%, $p< 0.001$) in hypothermic ALF rats (ALF-33) (Table 1).

Table 1
Effect of hypothermia on brain edema, ammonia and nitrite/nitrate levels in plasma and brain.

	Sham	ALF-6h	ALF-coma	ALF-33
Brain water (%)	78.04 ± 0.19	78.20 ± 0.12	80.83 ± 0.05*	78.31 ± 0.09†
Plasma ammonia (μM)	61.6 ± 8.7	728.7 ± 32.0*	1566 ± 72.1*	636.7 ± 57.0†
Brain ammonia (μM)	109.1 ± 6.5	1049 ± 77.8*	2067 ± 175.9*	682.3 ± 61.9†
Plasma nitrite/nitrate (μmol/L)	11.37 ± 0.61	17.3 ± 0.63*	17.4 ± 0.90*	12.6 ± 0.80†
Brain nitrite/nitrate (μmol/g)	1.17 ± 0.09	1.18 ± 0.10	1.76 ± 0.11*	1.22 ± 0.11††

Percentage of water content of cerebral cortex, plasma and brain ammonia and nitrite/nitrate levels in sham-operated controls (sham), ALF rats 6 h post-HAL (ALF-6 h), at coma stage of encephalopathy (ALF-coma) and in hypothermic ALF rats (ALF-33). Data represent mean ± S.E.M. of n=10 animals per treatment group. *$p < 0.001$ vs. sham; †$p < 0.001$ vs. ALF-coma; ††$p < 0.01$ vs. ALF-coma.

3.2. Effect of mild hypothermia on nitrite/nitrate and ammonia levels in plasma and brain of ALF rats

Plasma ammonia levels were increased 11.8-fold ($p < 0.001$) 6 h after HAL (ALF-6 h) and 25.4-fold ($p < 0.001$) in comatose ALF rats (ALF-coma) (Table 1). Brain ammonia levels were increased 9.6-fold ($p < 0.001$) 6 h after HAL and 18.9-fold ($p < 0.001$) at coma stages of encephalopathy (Table 1). Mild hypothermia reduced plasma ammonia levels by 59.3% ($p < 0.001$) and brain ammonia levels by 67.0% ($p < 0.001$). Nitrite/nitrate levels in plasma were elevated 1.5-fold 6 h after HAL and remained at the same levels in comatose ALF rats ($p < 0.001$). Nitrite/nitrate levels in plasma were significantly reduced by 27.6% ($p < 0.001$) due to mild hypothermia (Table 1). Nitrite/nitrate levels in brain were elevated 1.5-fold in comatose ALF rats but were unchanged 6 h after HAL. Mild hypothermia reduced brain nitrite/nitrate levels by 30.7% ($p < 0.01$) (Table 1). Plasma ammonia levels in rats maintained at either 37°C or 33°C were not significantly altered when measured at coma stage of encephalopathy.

Table 2
Effect of hypothermia on the progression of encephalopathy.

Progression of encephalopathy	Normothermic	Hypothermic
Time to loss of righting reflexes (h)	9.1 ± 0.3	14.4 ± 0.4*
Time to loss of corneal reflexes (h)	13.1 ± 0.4	18.4 ± 0.4*

Time to precoma (loss of righting reflexes) and coma (loss of corneal reflexes) stages of encephalopathy in normothermic and hypothermic ALF rats. Data represent mean ± S.E.M. of n = 10 animals per treatment group. *$p < 0.001$.

3.3. Mild hypothermia inhibits upregulation of HO-1, iNOS and eNOS expression in ALF rat brain

HO-1 mRNA level in brain was elevated 1.3-fold ($p < 0.01$) 6 h post-HAL and 1.7-fold ($p < 0.001$) in comatose ALF rats compared to sham-operated controls (Fig. 1). HO-1 protein expression was increased 1.2-fold ($p < 0.05$) 6 h post-HAL and 1.6-fold ($p < 0.001$) at coma stage of encephalopathy compared to sham-operated controls (Fig. 2). Mild hypothermia reduced HO-1 mRNA expression by 23.9% ($p < 0.001$) and HO-1 protein expression by 22.3% ($p < 0.001$). nNOS mRNA expression in brain was not statistically different between ALF rats and sham-operated controls (data not shown). However, iNOS and eNOS mRNA levels were elevated 5.0-fold and 1.8-fold, respectively, 6 h post-HAL ($p < 0.001$) and were further increased 9.0-fold and 2.7-fold ($p < 0.001$) in comatose ALF rats (Figs. 3 and 4). Mild hypothermia

decreased iNOS mRNA levels by 69.9% ($p < 0.001$) and eNOS mRNA expression by 24.8% ($p < 0.01$).

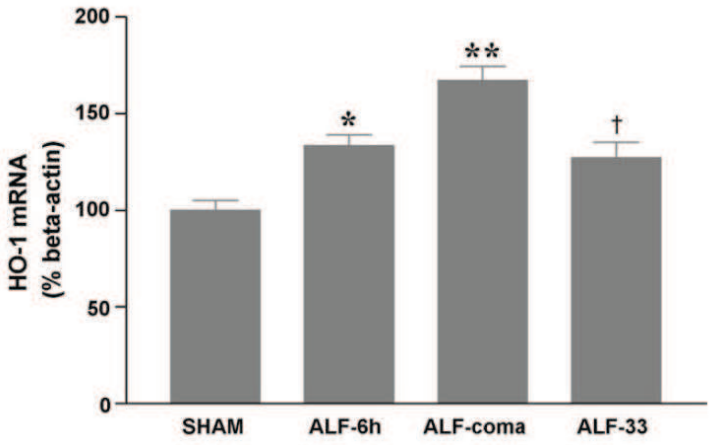

Fig. 1. Upregulation of brain HO-1 mRNA expression is attenuated by mild hypothermia in the cerebral cortex of ALF rats. Expression of HO-1 mRNA in the cerebral cortex of sham-operated controls (sham), ALF rats 6 h post-HAL (ALF-6h), ALF rats at coma stage of encephalopathy (ALF-coma) and hypothermic ALF rats (ALF-33). Data represent mean ± S.E.M. of n=10 animals per experimental group. $^{*}p < 0.01$ vs. sham; $^{**}p < 0.001$ vs. sham; †$p < 0.001$ vs. ALF-coma.

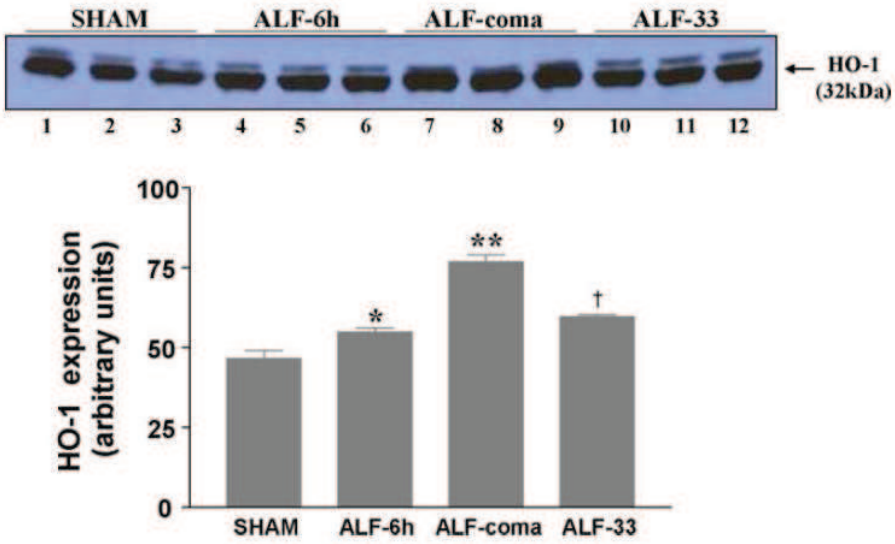

Fig. 2. Upregulation of brain HO-1 protein expression is attenuated by mild hypothermia in the cerebral cortex of ALF rats. Expression of HO-1 protein in the cerebral cortex of sham-operated controls (sham) (lanes 1–3), ALF rats 6 h post-HAL (ALF-6 h) (lanes 4–6), ALF rats at coma stage of encephalopathy (ALF-coma) (lanes 7–9) and hypothermic ALF rats (ALF-33) (lanes 10–12). Data represent mean ± S.E.M. of n=10 animals per experimental group. $^{*}p < 0.05$ vs. sham; $^{**}p < 0.001$ vs. sham; †$p < 0.001$ vs. ALF-coma.

4. Discussion

Results of the present study demonstrate that ALF resulting from hepatic devascularization leads to selective increases in expression of the gene coding for inducible and endothelial isoforms of NOS as well as for heme oxygenase-1 (HO-1) in brain. The magnitude of induction, in all cases, was a function of the duration of

liver ischemia, the presence of severe encephalopathy (coma), cerebral edema and of blood and brain ammonia concentrations. Increased iNOS and eNOS isoform gene expression was accompanied by significant increases in brain concentrations of nitrite/nitrate (NOx) confirming that the increases in NOS isoform expression caused by ALF were sufficient to cause increased NO production. Further studies are required in order to ascertain the precise cellular localization of the iNOS increases. Previous studies in a hepatotoxin model of ALF had provided evidence for oxidative stress in brain. Such evidence consisted of increased lipid peroxidation [491] and decreases in the GSH/GSSG ratio [492, 493] in the brains of these animals. However, a third study using a partial hepatectomy model of ALF failed to observe any significant changes in ascorbate status in this model [494], a finding that could be attributed to the relative early stage of ALF used in this study.

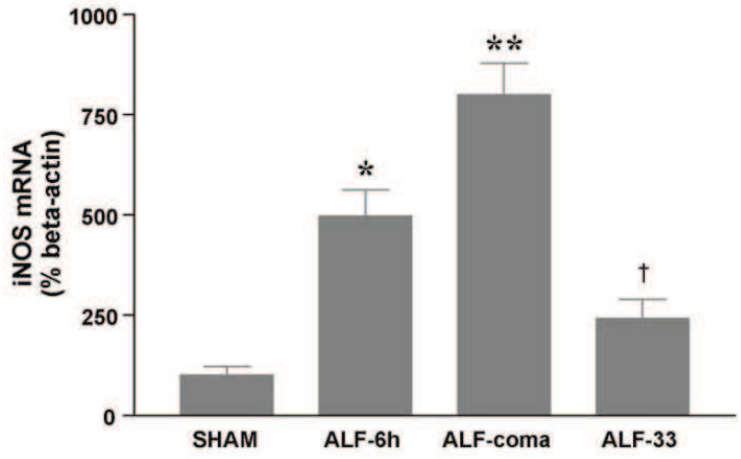

Fig. 3. Upregulation of brain iNOS mRNA expression is attenuated by mild hypothermia in the cerebral cortex of ALF rats. Expression of iNOS mRNA in the

cerebral cortex of sham-operated controls (sham), ALF rats 6 h post-HAL (ALF-6h), ALF rats at coma stage of encephalopathy (ALF-coma) and hypothermic ALF rats (ALF-33). Data represent mean ± S.E.M. of n=10 animals per experimental group. $^{*}p < 0.001$ vs. sham; $^{**}p < 0.001$ vs. sham; †$p < 0.001$ vs. ALF-coma.

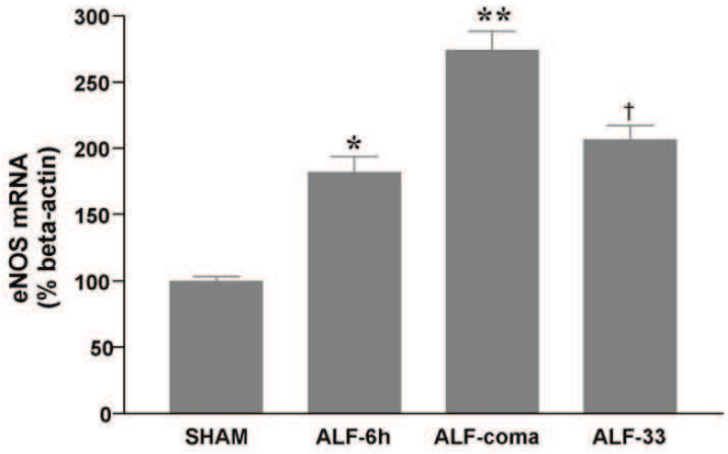

Fig. 4. Upregulation of brain eNOS mRNA expression is attenuated by mild hypothermia in the cerebral cortex of ALF rats. Expression of eNOS mRNA in the cerebral cortex of sham-operated controls (sham), ALF rats 6 h post-HAL (ALF-6h), ALF rats at coma stage of encephalopathy (ALF-coma) and hypothermic ALF rats (ALF-33). Data represent mean ± S.E.M. of n=10 animals per experimental group. $^{*}p < 0.001$ vs. sham; $^{**}p < 0.001$ vs. sham; †$p < 0.001$ vs. ALF-coma.

The precise cause of oxidative/nitrosative stress in brain of ALF in unclear. However, there is a great deal of indirect evidence to suggest that ammonia plays a role. Mice with hyperammonemia resulting from dietary loading display evidence

of lipid peroxidation in brain [495] and exposure of cultured astrocytes to concentrations of ammonia equivalent to those encountered in brain in ALF results in the generation of ROS [99] accompanied by decreased levels of GSH. Lipid peroxidation and increased production of nitric oxide have been reported in the brains of animals with portacaval shunts administered ammonia sufficient to produce brain edema [93]. The notion of a link between increased brain ammonia and nitrosative stress in ALF is strengthened by the finding of the present study of a correlation between brain ammonia increases and increased expression of NOS isoforms and HO-1 as a function of the presence of encephalopathy and brain edema in hepatic devascularized rats. In vitro studies suggest that ammonia-induced oxidative/nitrosative stress leads to cell swelling and involves alterations of the MPT complex and/or impaired cellular energy metabolism [496]. Whether or not similar mechanisms occur in ALF awaits further studies. Evidence against a unique role of ammonia generated oxidative/nitrosative in the pathogenesis of HE are the rather poor correlations observed in the present study between brain concentrations of ammonia and nitrite/nitrate levels as well as those between brain nitrite/nitrate levels and severity of encephalopathy clearly indicating that additional mechanisms could also be implicated.

Potential consequences of oxidative/nitrosative stress in ALF involve modifications of key brain proteins. For example, portacaval anastomosis results in nitration of glutamine synthase [497], an astrocytic enzyme uniquely responsible for removal of ammonia in the brain and ^{13}C NMR studies revealed decreased

capacity for brain glutamine synthesis in hepatic devascularized rats [115]. A second target protein is the astrocytic glutamate transporter EAAT-2 (formerly GLT-1) which is known to be inactivated by oxidative stress [258]; GLT-1 expression is significantly reduced in the brains of hepatic devascularized rats [262] and in rats with toxic liver injury [263]. Ammonia results in inactivation of the tricarboxylic cycle enzyme alphaketoglutarate dehydrogenase [168], an effect which has been attributed to the generation of ROS [498].

Further evidence that oxidative/nitrosative stress is implicated in the pathogenesis of brain edema and encephalopathy in ALF is provided by the findings of the present study where mild hypothermia (33°C) sufficient to delay encephalopathy and prevent brain edema significantly attenuates the increases in expression of HO-1 and NOS isoforms with consequent reduction of brain levels of nitrites/nitrates. Mild hypothermia also resulted in a significant attenuation of the increased brain concentrations of ammonia that are characteristic of ALF. Such action could result from decreased blood–brain barrier ammonia transfer, improvement in hepatic function or modulation of CBF [314, 446]. Decreased expression of oxidative stress markers could result, at least in part, from the lowering of brain ammonia concentrations. Mild hypothermia is currently in use in the management of ALF where beneficial effects on both hepatic and brain function have been described [39, 314, 446, 447]. Findings of the present study suggest that one of the beneficial effects of mild hypothermia in relation to the cerebral consequences of ALF relates to its potential to reduce oxidative/nitrosative stress-related mechanisms.

Acknowledgements

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Article 4:

Minocycline attenuates oxidative/nitrosative stress and cerebral complications of ALF in rats

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Abstract

In the present study, the effects of minocycline on progression of encephalopathy and brain edema in rats with acute liver failure (ALF) resulting from hepatic devascularization were studied in relation to the antioxidant action of the drug. ALF rats were sacrificed at precoma and coma stages of encephalopathy along with their appropriate sham-operated controls. Minocycline-treated ALF rats were sacrificed in parallel with comatose vehicle-treated ALF controls. Microglial activation was assessed using CD11b/c (OX-42) immunohistochemistry. Nitrite/nitrate levels in plasma and brain were measured using the Griess reaction. Expression of nitric oxide synthase (NOS) isoforms and heme oxygenase-1 (HO-1) were measured using real-time quantitative PCR and Western blot analysis. Increased nitrite/nitrate levels were observed in the plasma of ALF rats at coma stage of encephalopathy compared to sham-operated controls. Increased expression of HO-1 mRNA and protein was observed in the frontal cortex of ALF rats at both precoma and coma stages of encephalopathy. Significant increases in expression of endothelial (eNOS) and inducible (iNOS) isoforms of NOS mRNA and protein occurred only at coma stages of encephalopathy accompanied by increased brain nitrite/nitrate concentrations. As expected, minocycline attenuated microglial activation as confirmed by decreased OX-42 immunoreactivity, normalized nitrite/nitrate levels in brain and significantly attenuated HO-1, eNOS and iNOS expression. These results indicate that the beneficial effect of minocycline on the neurological complications of ALF is mediated, at least in part, by reduction of oxidative/nitrosative stress.

Keywords: Hepatic encephalopathy; Acute liver failure; Nitric oxide synthase; Oxidative stress; Brain edema; Minocycline

1. Introduction

Hepatic encephalopathy (HE) and brain edema leading to intracranial hypertension are serious neurological complications of ALF. Hyperammonemia is a critical factor in the pathogenesis of the central nervous system complications of ALF [79] and cerebral herniation in ALF patients has been shown to correlate with arterial ammonia concentrations [26]. However, it appears that other factors may also contribute to the cerebral complications of ALF. In particular, microglial activation and brain inflammation are correlated with the progression of encephalopathy and the onset of brain edema in rats with ALF resulting from hepatic devascularization [499]. Moreover, increased expression of the endothelial and inducible isoforms of nitric oxide synthase as well as heme oxygenase-1 have been reported in this model [500] suggesting that oxidative/nitrosative stress also plays a significant role in the pathogenesis of the cerebral complications of ALF.

Minocycline, a semisynthetic tetracycline with potent antimicrobial properties is known to limit microglial activation in a wide range of neurodegenerative conditions [463] and a previous study showed that it also delays progression of encephalopathy and the onset of brain edema in ALF rats [501]. It has been proposed that minocycline has antioxidant

properties [502]. Consequently, the objective of the present study was to assess the protective action of minocycline on oxidative/nitrosative stress in the brains of rats with ALF. Nitrite/nitrate levels as well as expression of heme oxygenase-1 (HO-1) and nitric oxide synthase (NOS) isoforms were measured in brain as a function of encephalopathy and brain edema severity using a well-characterized animal model of ALF, the hepatic devascularized rat [462].

2. Materials and methods

2.1. Hepatic devascularization and treatments

Adult male Sprague–Dawley rats (200–250 g) purchased from Charles River (Saint-Constant, Quebec, Canada) were routinely tested for common pathogens and were free of infection at the onset of surgery. Animals were anesthetized with isoflurane, and an end-to-side portacaval anastomosis was performed according to the guidelines of Lee and Fisher (1961)[448]. Briefly, rats underwent a laparotomy, the inferior vena cava and portal vein were isolated and clamped using an anastomosis clamp (Roboz Instruments Inc., Washington DC) and an elliptical portion 1.5 times the diameter of the portal vein was removed. The portal vein was ligated and cut, and an end-to-side anastomosis was performed under a dissecting microscope. Total surgery time was <30 min. Sham-operated control rats ($n = 10$), matched for weight, were similarly anesthetized and the inferior vena cava was clamped for 20 min. Following surgery, all animals were individually housed with free access to food and water under constant

conditions of temperature, humidity, and light cycles. Twenty-four hours after portacaval anastomosis, rats were reanesthetized and subjected to hepatic artery ligation (HAL). Following HAL, arterial blood glucose levels were monitored and glucose was administered subcutaneously as needed to maintain normoglycemia. Body temperature was monitored every 15 min and maintained at 37 ± 0.5 °C by means of heating pads. A group of animals (n = 10) was sacrificed 6 h following HAL (ALF-6 h) and a second group (n = 10) was sacrificed at coma stage of encephalopathy (ALF-coma) (defined as the loss of corneal reflexes). In a second set of experiments, a group (n = 10) of ALF animals (ALF-mino) were administered minocycline (22.5 mg/kg twice daily; i.p.) on days −2, −1 and day 0 of HAL operation. Control ALF rats (n = 10) were given equivalent volumes of saline. Minocycline-treated animals were sacrificed in parallel with comatose saline-treated ALF controls. Brains were rapidly removed, dissected on ice and were immediately frozen in isopentane. All tissues were stored at −70 °C until use. All the above surgical methods were conducted in accordance with the Guidelines of Canadian Council of Animal care and were approved by Animal Research Committee at Saint-Luc Hospital (C.H.U.M.).

2.2. Brain water measurement

Brain water was quantitated by the wet weight/dry weight method. Half of the brain was weighed before and after 48 h incubation in a 120 °C oven. Water content of the brain samples are expressed as percentage of water content according to the following equation: %water = (wet weight − dry weight)/wet weight × 100.

2.3. Ammonia measurements

Plasma and brain ammonia concentrations were determined using a commercial ammonia test kit (Sigma–Aldrich, St. Louis, MO) based on the enzymatic method using the glutamate dehydrogenase reaction.

2.4. Nitrite/nitrate levels

Nitrite/nitrate levels in brain and plasma were determined using Griess reagent [490]. Brain tissue were homogenized in water and diluted to 5 μg/μl. Brain and plasma samples (100 μl) were diluted 2-fold in water, deproteinized by adding 10 μl of 30% $ZnSO_4$ and centrifuged at 10,000 × *g* for min. To reduce nitrate to nitrite, the supernatant was incubated for 24 h at room temperature with 0.5 g of cadmium that were washed sequentially in 0.1 M hydrochloric acid and 0.1 M ammonium hydroxide before use. Samples (100 μl) or serial dilution of $NaNO_2$ standard (linear range 0–100 μM) were applied to a microtiter plate well. Total nitrite in each sample was then determined by adding 50 μl Griess reagent 1 (1% sulfanilamide in 1N HCl), followed by Griess reagent 2 (0.1% N-(1-napthyl)-ethylenediamine). The mixture was incubated for 10 min in the dark. Optical density was measured spectrophotometrically at 540 nm. Nitrate/nitrite levels were expressed as micromoles per gram tissue protein or micromoles per liter.

2.5. Immunohistochemistry

Animals were deeply anesthetized with pentobarbital (60 mg/kg; i.p.). After being transcardially perfused with 240 ml ice-cold saline followed by 240 ml neutral-buffered formalin (containing 4% formaldehyde, 0.5% sodium phosphate buffer, pH 7.0), brains were removed, post-fixed in 10% formalin at 4 °C for 12 h and transferred into ice-cold phosphate-buffered saline (PBS) solution for storage. 50 μm thick coronal sections were obtained using a vibratome from −4.0 to −5.5 mm relative to the bregma according to the rat brain atlas of [450]. Sections were incubated with 0.3% hydrogen peroxide in PBS for 10 min to block endogenous peroxidase activity. Sections were blocked with 2% horse serum and 0.5% Triton X100 and incubated at 4 °C overnight with mouse anti-CD11b/c (OX-42) (1/1000) (Cedarlane Laboratories, Burlington, NC). After washing with PBS, sections were incubated for 1 h with horse anti-mouse biotinylated secondary antibody (1/100) (Vector Laboratories, Burlingame, CA) and thereafter with Vectasain ABC reagent (Vector Laboratories). OX-42 immunoreactivity was detected by incubation with 3-3'-diaminobenzidine containing urea-hydrogen peroxide (Sigma–Aldrich). The sections were mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, CA), dehydrated stepwise in ethanol and xylene and coverslip with Permount (Fisher Scientific). Sections without primary antibodies were used as negative controls and showed no immunoreactivity.

2.6. Western blot analysis

Brain samples from frontal cortex were homogenized in ice-cold RIPA buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Sigma–Aldrich) and centrifuged at 12,000 × *g* for 45 min. Protein concentrations were measured using a DC Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins (100 μg) were solubilized in Laemmli buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol; 2% SDS, 10% dithiothreitol, 0.1% bromophenol blue) and boiled for 10 min. Proteins were resolved by 7–10% denaturing SDS-polyacrylamide gels and transferred overnight to PVDF membranes (Bio-Rad Laboratories) The filters were blocked for 1 h in Tris-buffered saline (TBS) containing 5% dry milk and 0.1% Tween 20, then incubated for 1 h with rabbit anti-HO-1 (1/700, Assay Design, Ann Arbor, MI), rabbit anti-iNOS (1/250, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-eNOS (1/250, BD Biosciences, Franklin Lakes, NJ), or mouse anti-beta-actin (1/10000, Sigma–Aldrich). The blots were subsequently probed with goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antiserum (PerkinElmer, Waltham, MA) diluted 1/10,000 in the same buffer. After extensive washing with TBS, the peroxidase activity was detected by chemiluminescence using the ECL detection system (Amersham, Arlington Heights, IL). Intensity of the bands was measured by densitometry using quantified using Quantity-One software (Bio-Rad Laboratories). Results are expressed as percentage of the housekeeping protein beta-actin to normalize for loading variation.

2.7. Real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Animals were transcardially perfused with 240 ml ice-cold saline to remove residual blood in the brain. Total RNA was isolated from rat brain cortex using the Trizol reagent (Invitrogen Ltd., Carlsbad, CA), according to the manufacturer's instructions. The cDNA was synthesized using thermoscript RT-PCR system (Invitrogen). Expression levels were assessed by real-time PCR in a RotorGene 3000™ Real time DNA detection system (Corbett Life Science, Sydney, Australia) with the Quantitect SYBRGreen I PCR kit (Quiagen, Valencia, CA). Oligonucleotide primers (Invitrogen) were designed using the primer3 program [451] at http://www.primer3.sourceforge.net/ and selected to include at least one intron. The specificity of the oligonucleotide primers was verified using the program BLASTN from the National Center for Biotechnology Information (NCBI, Bethesda, MD). For each primer pair, the amplified cDNA fragments were analyzed by agarose gel to confirm the absence of the intron and of any nonspecific PCR products. The forward and reverse oligonucleotide primer sequences were 5'-CCACAGCTGAGAGGGAAATC-3' and 5'-TCTCCAGGGAGGAAGAGGAT-3' for β-actin (GeneBank accession # V01217); 5'-TCAACAGTGGGAGCAAAGC-3' and 5'-CTTTGTGCGATTTGCCATC-3' for nNOS (GenBank accession # U67309); 5'-GATCCAGTGGGGGAAACT G-3' and 5'-GCCTCGGTTTGTTGCATACT-3' for eNOS (GenBank accession # AB176831) 5'-CCAAGGTGACCTGAAAGAGG-3' and 5'-TTGATGCTTGTGACTCTTAGGG-3' for iNOS (GenBank accession # U26686); 5'-GAGCCAGCCTGAACTAGCC-3' and 5'-GATGTGCACCTCCTTGGTG-3' for HO-1 (GenBank accession # J02722). A relative quantification was performed by comparing the threshold cycle values of

samples with serially diluted standards. Expression levels were normalized to the housekeeping gene β-actin.

2.8. Statistical analysis

All data are expressed as the mean ± SEM and statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis. A probability of $p < 0.05$ was chosen to establish significance between the groups. Data were analyzed by using Prism 4.0 software (Prism 4.0, San Diego, CA).

3. Results

3.1. Minocycline attenuates microglial activation and delays the progression of neurological complications in ALF rats

Following HAL, rats developed a progressive encephalopathy, characterized by lethargy (evident 6 h after HAL) progressing to loss of righting and corneal reflexes and coma. Sham-operated rats exhibited normal behavior and neurological reflexes throughout the period of the experiments. Brain water content was not significantly different between sham-operated rats and ALF rats 6 h after HAL (ALF-6 h) but was significantly elevated in ALF rats at coma stage (sham vs. ALF-coma: 78.09 ± 0.07% vs. 80.78 ± 0.1%, $p < 0.001$) (Table 1). Brain edema in ALF rats is correlated with microglial activation as shown by increased OX-42 immunostaining in frontal cortex

and other brain regions [499]. Minocycline attenuated microglial activation as shown by decreased OX-42 immunoreactivity (60.1%, $p < 0.001$) in the frontal cortex of ALF rats (Fig. 1) and concomitantly delayed progression of encephalopathy with longer times to precoma (loss of righting reflexes) (saline vs. minocycline: 9.1 ± 0.3 h vs. 13.1 ± 0.5 h, $p < 0.001$) and coma (loss of corneal reflexes) (saline vs. minocycline: 13.1 ± 0.4 h vs. 15.8 ± 0.4 h, $p < 0.001$). As previously reported, brain water content was also significantly ($p < 0.001$) reduced in minocycline-treated (79.38 ± 0.15%) ALF rats (Table 1) [501].

Table 1. Effect of minocycline on brain edema, ammonia and nitrite/nitrate levels in plasma and brain.

	Sham	ALF-6h	ALF-coma	ALF-mino
Brain water (%)	78.09 ± 0.07	78.23 ± 0.14	80.78 ± 0.10*	79.38 ± 0.15†
Plasma ammonia (μM)	61.6 ± 8.7	728.7 ± 32.0*	1566 ± 72.2*	719.5 ± 43.2†
Brain ammonia (μM)	109.1 ± 6.5	1049 ± 77.8*	2067 ± 175.9*	1219 ± 53.7†
Plasma nitrite/nitrate (μmole/L)	11.37 ± 0.61	17.3 ± 0.63*	17.4 ± 0.90*	17.05 ± 0.78
Brain nitrite/nitrate (μmole/g)	1.17 ± 0.09	1.18 ± 0.10	1.76 ± 0.11*	0.98 ± 0.07†

Water content of cerebral cortex, plasma and brain ammonia and nitrite/nitrate levels in sham-operated controls (sham), ALF rats 6 h post-HAL (ALF-6 h), at coma stage of encephalopathy (ALF-coma) and in minocycline-treated ALF rats (ALF-mino). Data represent mean ± SEM of $n = 10$ animals per treatment group. * $p < 0.001$ vs. sham; † $p < 0.001$ vs. ALF-coma.

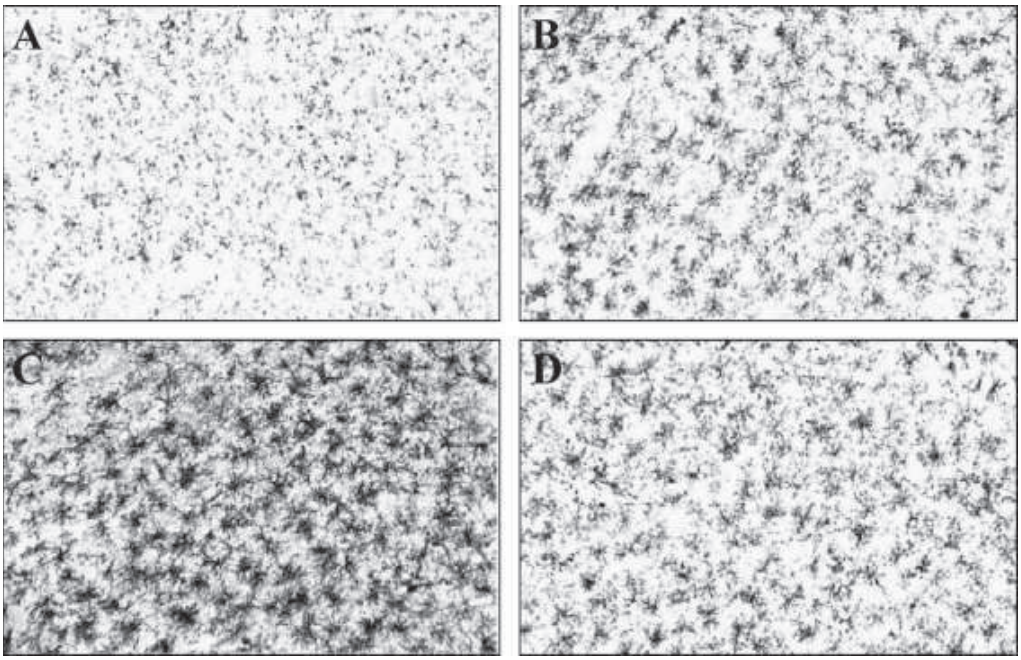

Fig. 1. Attenuation of microglial activation by minocycline in the brains of ALF rats Representative micrographs showing the effect of ALF on CD11b/c (OX-42) immunoreactivity in cerebral cortex of (A) sham-operated controls; (B) ALF rats 6 h post-HAL; (C) ALF rats at coma stage of encephlopathy; and (D) minocycline-treated ALF rats (magnification: 200×).

3.2. Effect of minocycline on nitrite/nitrate and ammonia levels in plasma and brain of ALF rats

Plasma ammonia levels were increased 11.8-fold ($p < 0.001$) 6 h after HAL and 25.4-fold ($p < 0.001$) in comatose ALF rats (ALF-37) (Table 1). Brain ammonia levels were

increased 9.6-fold ($p < 0.001$) 6 h after HAL and 18.9-fold ($p < 0.001$) in comatose ALF rats. Minocycline reduced plasma and brain ammonia levels by 54.0% ($p < 0.001$) and 41.0% ($p < 0.001$), respectively (Table 1). Nitrite/nitrate levels in plasma were elevated 1.5-fold 6 h after HAL and remained at the same levels in comatose ALF rats ($p < 0.001$) (Table 1). Nitrite/nitrate levels in brain were elevated 1.5-fold in comatose ALF rats but were not increased 6 h after HAL. Nitrite/nitrate levels in plasma were not changed by minocycline but were significantly reduced in brain at coma stages of encephalopathy (44.3%, $p < 0.001$) (Table 1).

3.3. Minocycline attenuates upregulation of HO-1, iNOS and eNOS expression in ALF rat brain

HO-1 mRNA levels in brain was elevated 1.3-fold ($p < 0.01$) 6 h post-HAL with further increases (1.7-fold, $p < 0.001$) in comatose ALF rats compared to sham-operated controls (Fig. 2a). Minocycline significantly reduced HO-1 mRNA expression by 32.7% ($p < 0.001$). HO-1 protein levels was increased 1.2-fold ($p < 0.01$) 6 h post-HAL and 1.6-fold ($p < 0.001$) at coma stages of encephalopathy (Fig. 2b). Minocycline significantly reduced HO-1 protein expression by 13.4% ($p < 0.01$).

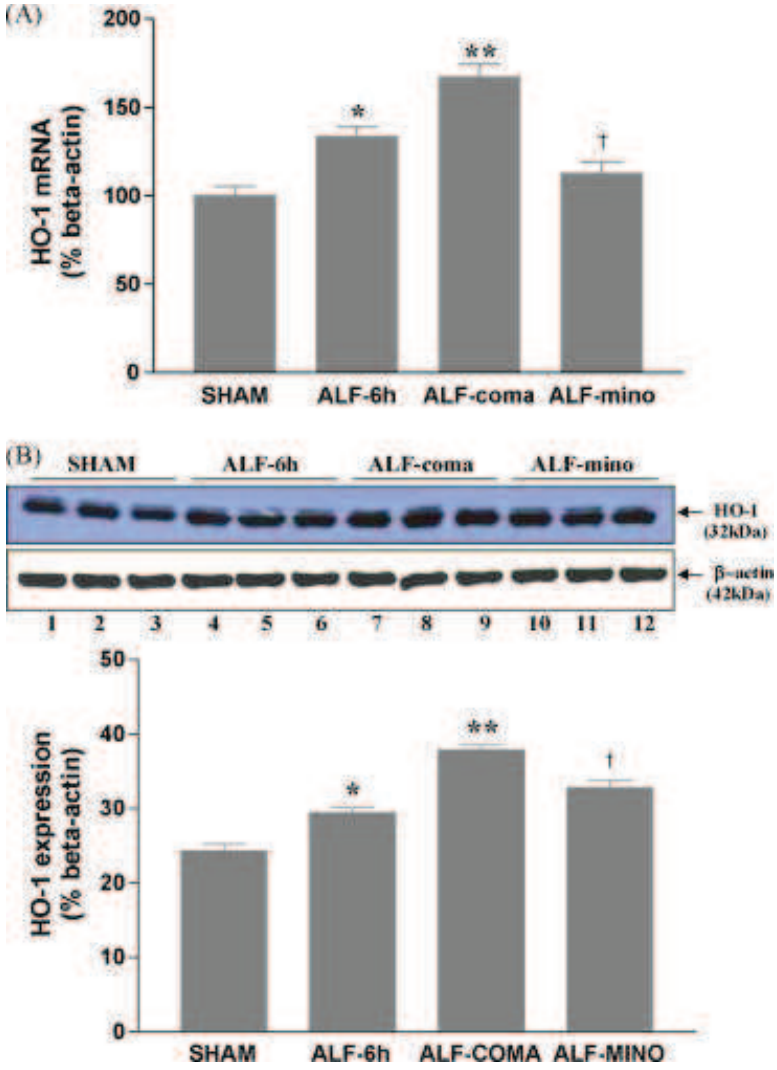

Fig. 2. Upregulation of brain HO-1 expression is attenuated by minocycline in the cerebral cortex of ALF rats. (A-) Expression of HO-1 mRNA and (B-) HO-1 protein in the cerebral cortex of sham-operated controls (SHAM) (lanes 1–3), ALF rats 6 h post-HAL (ALF-6 h) (lanes 4–6), ALF rats at coma stage of encephalopathy (ALF-coma) (lanes 7–9) and minocycline-treated ALF rats (ALF-mino) (lanes 10–12). Data represent mean ± SEM of $n = 10$ animals per experimental group. *$p < 0.01$ vs. sham; **$p < 0.001$ vs. sham; †$p < 0.001$ vs. ALF-coma; ††$p < 0.01$ vs. ALF-coma.

nNOS mRNA expression in brain was not statistically different between ALF rats and sham-operated controls (Fig. 3a). However, iNOS and eNOS mRNA levels were elevated 5.0-fold and 1.8-fold, respectively, 6 h post-HAL ($p < 0.001$) and were further increased 8.0-fold and 2.7-fold ($p < 0.001$) in comatose ALF rats (Fig. 3b and c). Minocycline significantly attenuated the iNOS and eNOS mRNA increases by 40.0% ($p < 0.01$) and 37.9% ($p < 0.001$), respectively. iNOS and eNOS protein levels were unchanged 6 h-post HAL but were significantly increased 1.7-fold ($p < 0.001$) and 1.4-fold ($p < 0.05$), respectively at coma stages of encephalopathy (Fig. 4 and Fig. 5). Minocycline significantly attenuated iNOS and eNOS protein expression by 41.5% ($p < 0.001$) and 42.5% ($p < 0.05$), respectively.

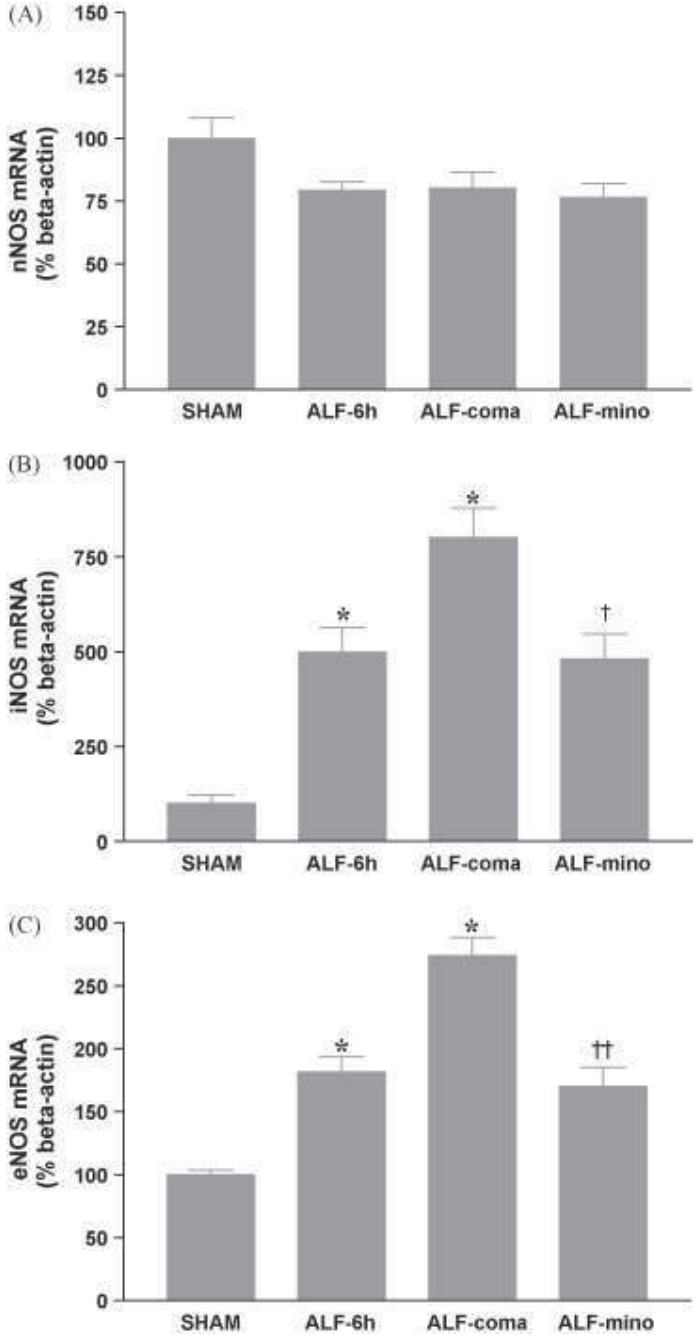

Fig. 3. Effect of minocycline on NOS isoform gene expression in the cerebral cortex of ALF rats. Expression of neuronal NOS (nNOS) (A-), inducible NOS (iNOS) (B-) and endothelial NOS (eNOS) (C-) mRNAs in the cerebral cortex of sham-operated controls

(SHAM), ALF rats 6 h post-HAL (ALF-6 h), ALF rats at coma stage of encephalopathy (ALF-coma) and minocycline-treated ALF rats (ALF-mino). Data represent mean ± SEM of $n = 10$ animals per experimental group. *$p < 0.001$ vs. sham; **$p < 0.001$ vs. sham; †$p < 0.01$ vs. ALF-coma; ††$p < 0.001$ vs. ALF-coma.

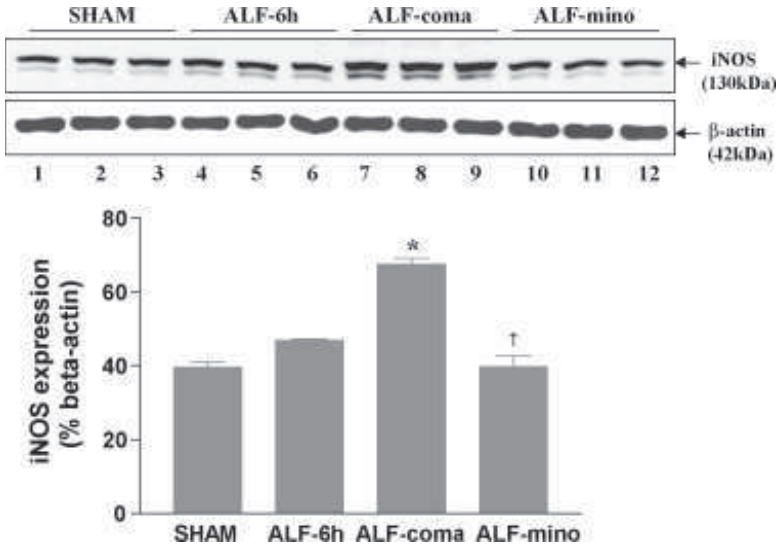

Fig. 4. Effect of minocycline on inducible NOS (iNOS) protein expression in the cerebral cortex of ALF rats. Expression of iNOS protein in the cerebral cortex of sham-operated controls (SHAM) (lanes 1–3), ALF rats 6 h post-HAL (ALF-6 h) (lanes 4–6), ALF rats at coma stage of encephalopathy (ALF-coma) (lanes 7–9) and minocycline-treated ALF rats (ALF-mino) (lanes 10–12). Data represent mean ± SEM of $n = 10$ animals per experimental group. *$p < 0.001$ vs. sham; †$p < 0.001$ vs. ALF-coma.

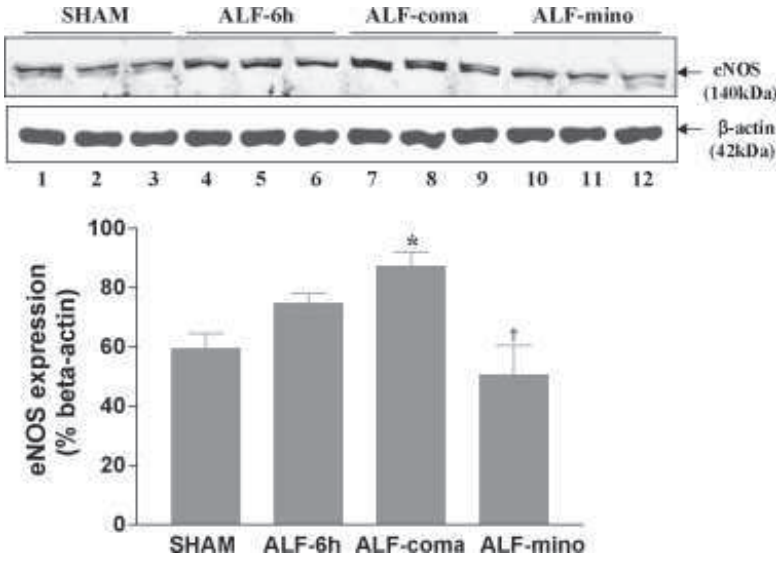

Fig. 5. Effect of minocycline on endothelial NOS (eNOS) protein expression in the cerebral cortex of ALF rats. Expression of eNOS protein in the cerebral cortex of sham-operated controls (SHAM) (lanes 1–3), ALF rats 6 h post-HAL (ALF-6 h) (lanes 4–6), ALF rats at coma stage of encephalopathy (ALF-coma) (lanes 7–9) and minocycline-treated ALF rats (ALF-mino) (lanes 10–12). Data represent mean ± SEM of $n = 10$ animals per experimental group. *$p < 0.05$ vs. sham; †$p < 0.05$ vs. ALF-coma.

4. Discussion

Results of the present study reveal that ALF resulting from ischemic liver failure leads to increased expression of the inducible and endothelial isoforms of NOS in brain. The magnitude of increase of NOS isoform expression was a function of the duration of liver ischemia and the presence of severe encephalopathy (coma) and cerebral edema. Furthermore, increased expression of iNOS and eNOS isoforms was accompanied by

increased brain concentrations of nitrites/nitrates confirming that the increases in NOS isoform expression caused by ALF were sufficient to lead to increased NO production. Previous studies in a model of ALF resulting from toxic liver injury also showed evidence of oxidative/nitrosative stress in brain [491] and [492].

The precise cause of oxidative/nitrosative stress in brain in ALF has not been definitively established. However, there is indirect evidence to suggest that ammonia plays a role. Hyperammonemic mice manifest evidence of lipid peroxidation in brain [495] and exposure of cultured astrocytes to low millimolar concentrations of ammonia (equivalent to those encountered in brain in ALF) results in the production of ROS [99]. ROS are produced in rat brain following acute ammonia intoxication [487] leading to glutathione depletion, lipid peroxidation and decreased activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase [503]. Moreover, lipid peroxidation and increased NO synthesis have been reported in the brains of animals with portacaval shunts administered ammonia sufficient to produce brain edema [93]. This notion of a pathophysiologic link between brain ammonia and nitrosative stress in ALF is strengthened by the findings of the present study of a correlation between brain ammonia increases, increased expression of NOS isoforms and nitrite/nitrate concentrations as a function of the presence of encephalopathy and brain edema.

Attenuation of increased circulating levels of ammonia in ALF by minocycline treatment could be the consequence, at least in part, of effects of the drug on gut

ammonia production. Further studies are warranted in order to address this possibility. Evidence that oxidative/nitrosative stress is implicated in the pathogenesis of brain edema and encephalopathy in ALF is provided by the findings of the present study where administration of minocycline led to a delay in onset of coma, prevented brain edema and also led to significant attenuation of the increases in expression of markers of oxidative/nitrosative stress (HO-1 and NOS isoforms) with consequent reduction of brain levels of nitrites/nitrates. On the other hand, the beneficial effect of minocycline on brain, but not circulating, nitrates/nitrites levels is consistent with the central anti-inflammatory properties of the drug. A previous study showed that minocycline had neuroprotective properties in mixed cultures of neural cells exposed to oxidative stress with a potency similar to that of vitamin E [502] and it was suggested that minocycline's antioxidant properties could be attributed to the presence of a substituted phenol ring in its chemical structure again, similar to that of vitamin E. It is conceivable that the beneficial effect of minocycline on oxidative/nitrosative stress also relates to its property to inhibit microglial activation. Under certain defined conditions such as exposure to excitotoxic agents, microglia may be activated and release NO [471]. These findings suggest that the beneficial effects of minocycline in relation to the cerebral consequences of ALF relates, at least in part, to its potential to reduce oxidative/nitrosative stress.

Acknowledgements

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CHAPTER 3

GENERAL DISCUSSION

1. Neuroinflammation plays a key role in the pathogenesis of HE and brain edema in ALF

1.1 Clinic and experimental evidence of inflammation in the pathogenesis HE and brain edema in ALF

Infection and inflammation are common features in ALF's natural history. Infection is documented in at least 80% of ALF patients [296], which leads to a rise of proinflammatory cytokines (TNF-α, IL-6 and IL-1β) in blood [297-300]. Besides coexisting infection, ALF itself is an inflammatory state, in which increased pro-inflammatory cytokines levels are observed [298, 301, 302], most obvious in patients with elevated ICP. The production of systemic pro-inflammatory cytokines might be attributed to the stimulation of the immune system by infection or mediators released from necrotic liver, or a direct release of cytokines from the necrotic liver.

In two large clinical studies of ALF, retrospective [303] or prospective [304], the presence of SIRS and/or infection was correlated with the severity of HE, increased ICP and high mortality rate. The results of these two studies support the view that inflammation plays a role in the development of brain edema and HE in ALF [305].

In a case report of an ALF patient with uncontrolled high ICP, hepatectomy caused a decrease of ICP and produced hemodynamic stability [306], associated with a reduction of circulating TNF-α, IL-6 and IL-1β [306], but without alteration of

arterial ammonia levels. This case provides evidence that necrotic liver-derived systemic proinflammatory cytokines exacerbate brain edema in ALF [306]. In a pig ALF model induced by hepatotoxin-amatoxin, co-treatment with LPS worsens and accelerates the progress of brain edema and HE [312].

In acute-on-chronic liver failure, inflammation and infection are important contributing mechanisms [307]. Similarly, the synergistic effect of inflammation on ammonia toxicity has been demonstrated in many chronic liver diseases [295, 308, 309]. Intestinal bacteria passing into lymph nodes and into blood might be possible pathogenic routes [310].

This increase in clinical and experimental evidence supports the concept that there is a synergistic effect between hyperammonia and inflammation in the pathogenesis of HE and brain edema in ALF.

1.2 Effects of systemic and central-derived cytokines on the development of HE and brain edema in ALF

In the multi-factorial hypothesis of the development of HE and brain edema in ALF, the central role of ammonia has not been questioned, based on extensive amounts of experimental and clinical evidence [504, 505]. The question is how the inflammation generates a synergistic harmful effect with ammonia during the pathogenesis of ALF.

Pro-inflammatory cytokines are large molecules, whose translocation from the circulation into the brain is constrained by the BBB. Because the BBB is generally regarded as intact in ALF patients, systemic cytokines are traditionally considered to be unable to affect the CNS directly. Emerging new experimental evidence suggests that systemic-derived and central-derived cytokines could affect brain via different pathways. Systemic inflammation might be able to send signals to the brain through a vagus nerve-dependent mechanism [506]. The activation of nucleus tractus solitarius from vagus nerve has been demonstrated in LPS peripherally injected animals [507]. Vagotomy inhibits cerebral IL-1b mRNA expression, induced by peripheral IL-1b stimulation [508]. The rapid signalling from the vagus nerve to the brain may explain the "sickness behaviour" during inflammation in ALF [509]. Systemic cytokines could also send signals to brain through stimulation of BBB endothelial cells that express IL-1β and TNFα receptors [510-512]. Through cytokine-binding and activation, endothelial cells produce secondary messengers and result in the intra-cerebral synthesis of NO and prostanoids [456, 511, 513, 514]. Perivascular microglia might be activated during systemic-derived cytokine stimulation [515]. This pathway could contribute to the observed changes of CBF and astrocytes in ALF (Figure 3).

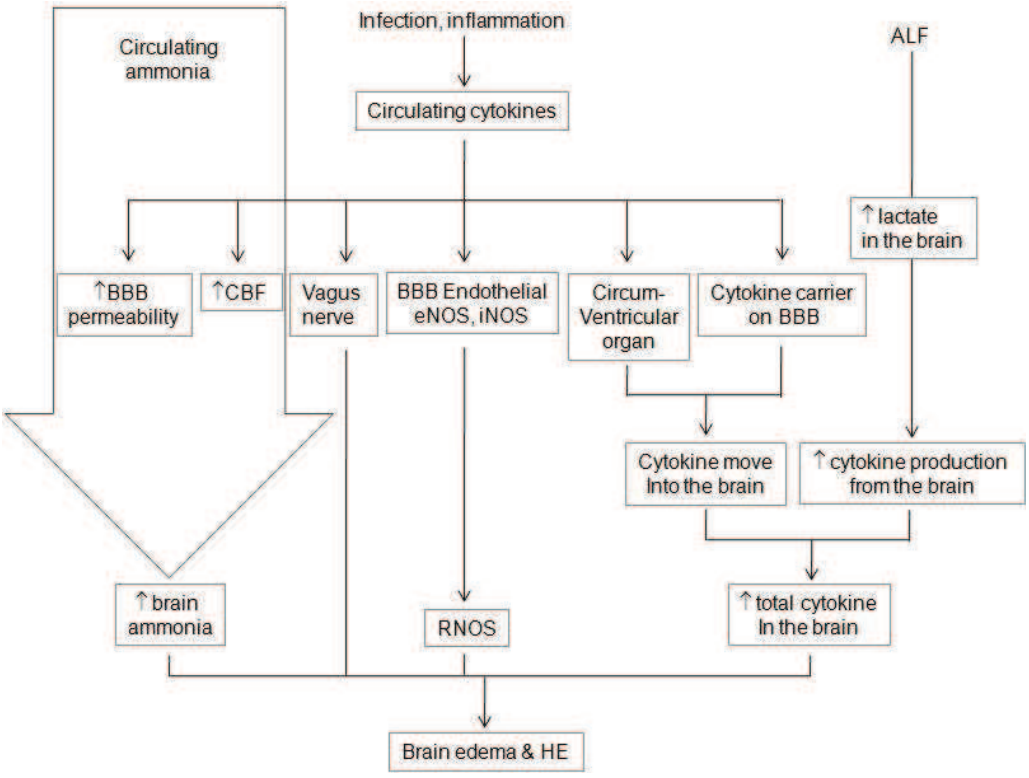

Figure 3: Potential mechanisms that link the peripheral and central inflammation to the development of cerebral edema and HE in ALF. The synergistic effect between inflammation and ammonia via BBB and CBF is noteworthy.

Systemic cytokines may also enter the CNS directly, through places devoid of a BBB, such as the circum-ventricular organs (the orgnum vasculosum of the lamina terminalis and area postrema) [456]. After crossing the blood vessel, cytokines diffuse into cerebral parenchyma, and stimulate astrocytes and microglia, to produce toxic mediators and exert their detrimental effects [516]. Systemic cytokines TNF-α, IL-1β, and IL-6 may also be able to cross the BBB via a saturable carrier-mediated transport mechanism [517-520].

A central mechanism of de novo cytokine synthesis has been proposed in human and experimental ALF. Increased cerebral efflux of IL-1β, TNFα and IL-6 was demonstrated in ALF patients with uncontrolled ICP [306, 521, 522], whereas such cytokines efflux can be reduced by hypothermia treatment to control high ICP [39, 311]. Results in the present thesis demonstrate unequivocally the direct evidence for brain-derived proinflammatory mechanisms in the pathogenesis of the neurologic complications of ALF. This evidence includes activation of microglia, as shown by increased immunoreactivity of the major histocompatibility complex marker, OX-42, together with the increased production of brain proinflammatory cytokines. Increased cytokine transcripts in the hepatic devascularized rat model of ALF were found to be selective in terms of their identity, the magnitude of the increase in their expression, and the timing of the increased expression in relation to the progression of ALF. Early increases in IL-6 mRNA levels were followed by more substantial increases in the expression of IL-1β and TNF-α and occurrence of severe encephalopathy and brain edema was associated with a generalized increase in the expression of all three cytokines [523].

This increased cerebral cytokine expression may be implicated in the altered gene expression found in HE. Altered expression of cerebral GFAP [208], the glutamate transporter EAAT-2 [262], the glucose transporter GLUT-1 [193], aquaporin IV [475] and the mitochondrial peripheral-type benzodiazepine receptor [217] have been demonstrated in experimental ALF animals, and found to be associated with

the development of brain edema. Although such protein modifications had previously been attributed to the toxic effects of ammonia in ALF, there is evidence to suggest that pro-inflammatory cytokines could also be implicated. Consistent with this notion, intracerebral administration of IL-1β leads to up-regulation of peripheral-type benzodiazepine receptor sites [476] while exposure of cultured astrocytes to IL-1β and TNF-α up-regulate aquaporin IV [477] and down-regulate GFAP [478] respectively. Moreover, high affinity transport of glutamate into cultured astrocytes is significantly impaired by exposure to IL-1β consistent with down-regulation of glutamate transporters [258, 479].

Systemic inflammation has detrimental effects on BBB permeability, other than BBB signal transduction. It has been suggested that circulating cytokines could alter BBB permeability in humans, probably by stimulating the vascular endothelium [344]. Systematic inflammation worsens clinical symptoms of HE in cirrhotic patients fed an ammoniagenic diet, which suggests an increased BBB permeability to blood-born toxins by inflammation [295]. Increased BBB permeability has also been demonstrated in endotoxin-induced inflammation in experimental ALF [345], and in naïve rats co-administration with LPS and ammonia, which results in increased CBF and ICP [327]. Furthermore, more obvious astrocyte swelling, along with anatomically intact BBB, has been demonstrated in LPS treated hyperammonemic rats, which indicates functional change of BBB, probably increasing BBB permeability to circulating toxins (ammonia) by systemic inflammation [346]. In addition, in vitro experiments have demonstrated various mechanisms on how different cytokines affect BBB. IL-1β

[459] and TNFα [459, 524, 525] have been shown to impair BBB integrity and function in endothelial cells. TNF-α and IL-6 increase ammonia diffusion across endothelial cells [458]. Furthermore, TNF-α and IFN-γ could compromise tight-junction integrity [526]. Incubation with TNF-*α* leads to astrocyte swelling in culture [527]. However, caution should be taken when translating in vitro results into in vivo interpretation.

Systemic inflammation might also induce elevated CBF in ALF. It has been recognized that increased CBF plays a role in the pathogenesis of brain edema and higher ICP in ALF patients [313, 528] (details in CBF section). Increased SIRS and pro-inflammatory cytokines have been shown to be associated with elevated CBF in ALF patients with higher ICP [294]. Hepatectomy, which decreases circulating pro-inflammatory cytokines, without change in blood ammonia, leads to a subsequent reduction in both CBF and ICP [306]. In a recent animal experiment, the synergistic effect of inflammation with hyperammonemia in producing increased CBF and ICP has been demonstrated in rats [327]. Inflammation significantly increases CBF and ICP, with an unaltered blood ammonia level [327], which suggests that observed brain edema and higher ICP is at least partially due to high CBF-induced increased ammonia extraction by the brain [529].

The various mechanisms mentioned above afford possible explanations for the concept of synergistic effects between inflammation and ammonia on the pathogenesis of brain edema and HE in ALF. However, the precise molecular and pathophysiologic mechanisms await further investigation. In view of the frequency of

infection and inflammation in ALF observed in the clinical setting, anti-inflammatory treatments affecting the different stages (up-stream and down-stream) in the rise of cytokines may offer new therapeutic approaches.

2. The role of RNOS in the pathogenesis of HE and brain edema in ALF

2.1 Evidence of oxidative/nitrosative stress in the pathogenesis of HE and brain edema

Alzheimer type II astrocytosis [530] is a typical pathology found in postmortem HE brain. The observation of increased lipofuscin pigment accumulation in these Alzheimer type II astrocytes indicates an enhanced oxidative stress which leads to raised lipid peroxidation [531, 532]. Although not many investigations on oxidative stress have been performed in human subjects with HE, animal experiments and cell culture systems have provided much evidence suggesting the involvement of RNOS (reactive oxygen and nitrogen species) in the pathogenesis of HE [98, 533].

Increased brain production of nitric oxide has been observed in different animal models, including ammonia-injected naïve rats [534], portacaval-shunted rats given ammonia infusions [93], rats with ischemic liver failure (Jiang et al, in press), as well as sepsis [535]. Increased brain generation of superoxide radical has also been demonstrated in ammonia-intoxicated animals [488, 503, 536, 537]. Hypothermia reduces free radical production [538], which may account for some of its beneficial

effects in HE. In addition, increased cerebral HO-1 expression has been found in hyperammoneamia animals, which indicates oxidative stress in the brain of these animals [325, 539, 540].

Nitric oxide and superoxide react to form a more toxic product peroxynitrite, which may modify proteins to generate a stable product - nitrotyrosine. Consistent with this notion, increased protein tyrosine nitration is observed in ammonia-administrated animals [378, 497]. Likewise, increased cerebral oxidative stress and protein tyrosine nitration are demonstrated in experimental animals administrated diazepam and endotoxin - two HE precipitation factors [541-544]. Using immunohistochemistry, the enhanced nitrotyrosine was found to occur with GS and GFAP (two astrocytic proteins), in brain samples from ammonia-, diazepam-, or endotoxin-administrated animals, indicating that protein tyrosine nitration is mainly in astrocytes [326, 378, 497, 543, 544].

Astrocyte swelling is a well known feature of HE. The role of RNOS in mediating the development of astrocyte swelling is supported by many experiments. Astrocyte swelling, with a reduction of myo-inositol, taurine and hypotaurine, has been observed in hydrogen peroxide-treated astrocyte cultures [545]. Astrocyte swelling could also be induced by ammonia treatment, with involvement of RNOS generation [99, 546], as well as by glutamate stimulation, in association with the production of RNOS [547, 548]. Furthermore, two groups of agents, GS inhibitors and NMDA receptor blockers, which prevent ammonia-induced protein tyrosine nitration, inhibit the clinical signs of

HE in ammonia-toxicated experimental animals [51, 90, 407, 488, 549-552]. Together, this evidence supports the hypothesis that RNOS and nitrotyrosine are involved in the development of HE and brain edema in ALF.

2.2 Mechanisms of increased RNOS production in HE

Several possible mechanisms have been proposed to explain the elevation of oxidative/nitrosative stress in HE: (1) increase of NOS1, NOS2 and NOS3 expression, (2) activation of NMDA receptors, (3) mitochondrial dysfunction, (4) decreased activities of antioxidant enzymes, and (5) astrocyte swelling. These mechanisms are not mutually exclusive. They likely represent either successive steps leading to RNOS generation or parallel pathways inducing RNOS production.

2.2.1 Increase of NOS1, NOS2 and NOS3 expression

Increased cerebral NOS1/nNOS (nitric oxide synthase) activity has been found in portacaval shunted rats [553]. And, increased cerebral NOS1/nNOS mRNA and protein expressions were also found in acute hyperammonemia animals [554], as well as portacaval-shunted rats with chronic hyperammonemia [555]. In investigating the role of ammonia as a causative factor, ammonia-treatment induces increased L-arginine uptake. L-arginine is the obligate precursor for NOS1/nNOS, in nerve terminal (synaptosomal) preparations [556] and in primary astrocyte cultures [557]. This ammonia-stimulated increase of L-arginine uptake occurs with

increased nNOS activities, in both in vitro and in vivo settings [553, 555]. In addition, the selective NOS1/nNOS inhibitor - nitroarginine - prevents several acute hyperammonemia-induced toxic effects in experimental animals [554]. However, nitroarginine and 1-2-trifluoromethylphenyl imidazole (TRIM), NOS1/nNOS inhibitors, did not prevent brain edema in portacaval shunted rats following ammonia administration [25], which indicates that either nNOS is not the only source of RNOS production in HE (which is evident by recent finding on iNOS and eNOS expression in ALF animals), or RNOS is not the major factor in HE pathogenesis.

Results of the present thesis demonstrate that ALF resulting from hepatic devascularization leads to selective increases in expression of the gene coding for inducible and endothelial isoforms of NOS (iNOS/NOS2 and eNOS/NOS3) in brain. The magnitude of induction, in all cases, was a function of the duration of liver ischemia, the presence of severe encephalopathy (coma), cerebral edema and of blood and brain ammonia concentrations. Increased iNOS and eNOS isoform gene expression was accompanied by significant increases in brain concentrations of nitrite/nitrate (NOx) confirming that the increases in NOS isoform expression caused by ALF were sufficient to cause increased NO production [337, 553]. In addition, increased iNOS protein expression, along with high nitrite production, has also been demonstrated in ammonia-intoxicated rats, as well as ammonia-treated astrocyte culutres [497]. In addition, increased nitrite release and iNOS protein expression was observed in LPS or cytokine treated astrocyte cultures [558]. Elevated cerebral eNOS

protein was also demonstrated in portacaval-shunted rats given ammonia treatment [559].

2.2.2 Activation of NMDA receptors

Consistent with an earlier report that hyperammonia-induced free-radical production may be mediated by NMDA-receptor activation [560], ammonia-intoxicated animals produce increased cerebral superoxide, NO and nitrotyrosine, in an NMDA receptor-dependent manner [378, 561]. Administration of NMDA receptor inhibitors, which block ammonia-induced astrocyte-swelling, reduce cerebral NO production and nitrotyrosine in ammonia-intoxicated animals [93, 407, 551, 561].

Evidence provided from in vitro experiments shows that NMDA activation leads to increased intracellular calcium concentration; and this increased intracellular calcium induces mitochondrial RNOS production, and iNOS expression and NO generation (through calcium-IkB pathway) [497, 551]. Similarly, NMDA receptor activation and increased intracellular calcium concentrations are also necessary steps in inducing increased astroglial nitrotyrosine by diazepam and TNF-α stimulation [543, 544]. However, further experiments are needed to prove the existence of this molecular pathway in vivo [562].

2.2.3 Mitochondrial dysfunction

Ammonia-induced mitochondrial malfunction [166] could be a source of increased ROS production [563]. Increased superoxide radical production from cerebral mitochondria has been demonstrated in acute ammonia-toxication [488, 503]. Increased RNOS production by ammonia could relate to the ammonia metabolite - glutamine, since GS inhibitors prevent ammonia-induced RNOS production, increased nitrotyrosine, and astrocyte-swelling in cultured astrocytes [78, 99, 497]. Increased free radical generation was observed in cultured-astrocytes treated with glutamine [130, 564]. And, ammonia released from glutamine hydrolysis was suspected, since PAG inhibition prevents free radical production [127, 128]. Astrocytic glutamine is mainly metabolized by mitochondrial PAG (in vitro setting), and glutamine hydrolysis leads to high mitochondrial ammonia, which impairs mitochondrial activity and increases ROS production. In addition, PTBR ligands induce free radical production in cultured astrocytes [565]; and since PTBR is located on mitochondrial membranes, and increased cerebral expression of PTBR is observed in ALF [566], this finding indicates that PTBR may play a role in RNOS production. Following further investigation, increased opening of the MPT pore, in the inner mitochondrial membrane, has been demonstrated in cultured astrocytes following exposure to ammonia [100], glutamine [129], PTBR ligands [567], or RNOS [568, 569]. Opening of the MPT pore causes mitochondrial dysfunction and increases ROS production.

2.2.4 Decreased activities of antioxidant enzymes

The antioxidant enzyme system (a defence line against RNOS) is compromised during HE. Reduced activities of cerebral superoxide dismutase, catalase and glutathione peroxidase were observed in rats following acute ammonia intoxication [503]. And, decreased antioxidant enzyme activities has also been demonstrated in sepsis [570], which has detrimental effects on astrocyte function [558]. Moreover, antioxidant administration has beneficial effects on hyperammonemic animals with HE [571].

2.2.5 Astrocyte swelling

Astrocyte swelling may itself induce RNOS production. Using hypo-osmotic-treated astrocyte cultures as a model, astrocyte swelling leads to increased RNOS production and high nitrotyrosine [572], which could be prevented by co-treatment with the NMDA receptor antagonist: MK-801 and EGTA [572]. Furthermore, increased intracellular calcium, induced by hypoosmotic situations, was inhibited by MK-801 [572], and increased nitrotyrosine, after hypoosmotic induction, was prevented by pre-treating astrocytes with MK-801, EGTA, a calmodulin antagonist W13, non-specific NOS inhibitors, SOD plus catalase, and uric acid [572]. These findings indicate that astrocyte swelling activates the NMDA receptor, increases intracellular calcium, enhances RNOS production, and subsequently increases nitrotyrosine levels [572].

Mechanisms involved in the NMDA receptor stimulation by swelling astrocyte are not clear. One possibility is astrocytic depolarization after hypoosmolarity [573], which leads to the deletion of the Mg^{2+} blockade on NMDA receptors [574]. Another

possibility is glutamate release from the swollen astrocyte, which could stimulate NMDA-R. RNOS production after astrocyte swelling could also result from increased release of ascorbate [575] and glutathione [576].

Moreover, as mentioned in the previous paragraph, increased RNOS levels could induce astrocyte swelling, and since swollen astrocytes could also enhance RNOS generation, then a vicious cycle could be established, with the potential to accelerate the progression of astrocyte swelling.

2.3 Astrocyte is the main cellular source of RNOS in brain in HE

The astrocyte has been considered as the main cellular source of cerebral RNOS production in HE. In one study, MSO, a GS inhibitor, prevented increased RNOS and nitrotyrosine formation in ammonia-treated cultured astrocytes [99, 497]. MSO also decreased cerebral production of NO and nitrotyrosine in ammonia-administrated animals [93, 561]. Because GS is an astrocyte specific protein, MSO experiments indicate that the astrocyte is closely involved in RNOS production. In cultured astrocytes, increased RNOS and nitrotyrosine formation has been observed following exposure to ammonia, PTBR ligands, and TNF-α [99, 497, 543, 544, 565]. In addition, ammonia exposure leads to increased argininosuccinate synthetase and lyase expression [577] and high arginine uptake [557] in cultured astrocytes, but not in neurons [577]. Ammonia and glutamine treatments lead to increased ROS and MPT production in astrocytes, but not neurons [100, 143]. Moreover, non-selective NOS

inhibition ameliorates hyperammonia-treated animals, but not by neuron-specific nNOS inhibition [25, 528, 536, 554]. These findings strongly support a key role for the astrocyte but do not exclude the role of microglia for RNOS production in HE.

2.4 RNOS in HE: possible mechanisms

How the generated RNOS and subsequent nitrotyrosine formation is involved in the pathogenesis of HE is not clear. Several molecular and pathophysiologic mechanisms have been proposed. Oxidation and nitrotyrosine formation could modify multiple proteins and affect their functions. In line with this view, nitrotyrosine residues have been found on PTBR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), GS, MAP-kinase and Erk-1 in ammonia-exposed astrocyte culture [497]. Increased PTBR oxidation leads to increased binding affinity of PTBR for its ligand PK11195 and increased inward transport of cholesterol into mitochondrial [578], which is involved in the alterations of cerebral neurosteroids synthesis, GABA receptor inhibition, and astrocytic energy metabolism [579, 580] (see more in PTBR section). Increased GAPDH oxidation could lead to its inactivation and proteasomal degradation [581] and impair energy metabolism in HE [582].

GS tyrosine nitration in ammonia-treated astrocytes is associated with its inactivation [497]. Similarly, tyrosine nitration of hepatic GS from LPS-treated rats and of sheep GS after peroxynitrite-treatment is associated with enzyme inhibition [544, 583, 584]. In contrast, giving the NMDA receptor antagonist (MK-801) [585] or the NOS

inhibitor (nitroarginine) [554] increases cerebral GS activity. And, preventing GS tyrosine nitration by epicatechin [586], partly restores GS activity [583]. These results support the view that GS activity is inhibited by tyrosine nitration. In liver failure, whether the modification of GS by tyrosine nitration occurs in only neurons, or in only astrocytes, or in both cell types, is waiting to be elucidated. But, reduced cerebral GS activity and decreased de novo cerebral glutamine synthesis capacity have been a consistent observation in liver failure [55, 587, 588]. Reduced GS enzyme activity decreases the capacity for ammonia removal in the brain.

Several studies suggest that RNOS could induce astrocyte swelling by influencing MPT function. Cyclosporin-A treatment prevents ammonia-induced astrocyte swelling, through the involvement of MPT pore [101, 129, 144]. In a recent experiment, PTBR was found to be involved in ammonia-induced RNOS production, unlock of MPT pore in cultured astrocytes, and subsequent astrocyte swelling [589].

Protein tyrosine nitration is observed in BBB-forming cerebral perivascular astrocytes in several animal models of HE [378, 497, 543, 544]. Several studies have shown that protein tyrosine nitration by peroxynitrite impairs BBB integrity [563]. In an experimental allergic encephalomyelitis (EAE) animal model, the increased BBB permeability was partially prevented by uric acid, a peroxynitrite scavenger [590-592]. The possible mechanism of increased BBB permeability by elevated nitrotyrosine modification may be attributed to functional alterations of substrate transport into astrocytes in HE.

The increased nNOS/iNOS/eNOS expression and increased NO generation may cause cerebral vasodilation and induce high CBF, a well-known phenomenon observed in ALF patients (see more in CBF section).

Some studies show that astrocytes are less sensitive than neurons to RNOS [593, 594]. Astrocyte-generated RNOS may impair nearby neurons in the brain. In one study, co-cultured peroxynitrite-treated astrocytes with embryonic motor neurons led to apoptosis of these neurons [593]. In another study, adding NO-producing astrocytes to cultured neurons makes these neurons vulnerable to glutamate treatment, and results in permanent damage to the respiratory chain and ATP reduction [595]. It is possible that astrocyte-produced RNOS may alter and impair neuronal function in HE.

The precise molecular pathways that link RNOS to HE remain to be elucidated. The synergistic action of ammonia, glutamate, benzodiazepines, hyponatremia and inflammatory cytokines in inducing RNOS formation from different mechanisms is worth noting. However, we need to keep the possibility open that protein tyrosine nitration might only be a marker of RNOS, and that other pathophysiologically more relevant mechanisms, induced by high RNOS production, may exist. Further studies are necessary to be carried out, in order to accurately assess the role of RNOS in the development of HE.

3. The involvement of microglia in the pathogenesis of HE and brain edema in ALF

3.1 General background of microglia

Microglia comprise nearly 10% of the cerebral cellular population, which function as resident mononuclear phagocytes of the brain [347, 348] and give differential response to various stimuli [349]. Microglia share lineage-related phenotypes with bone-marrow-derived monocytes and macrophages [350]. As macrophages, activated microglia could eliminate deleterious debris by phagocytosis and support tissue survival by secreting neurotrophic factors at cerebral injury sites. Microglia are considered to be major cell types in CNS responsible for cytokine production [351-355]. It has been shown that activated microglia can generate various cytotoxic substances in *in vitro* settings, its release has been implicated in the development of several neuroinflammatory/neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, AIDS dementia, multiple sclerosis, amyotrophic lateral sclerosis, traumatic brain injury [356], stroke [357], and Wernicke's encephalopathy [358].

3.2 Mechanisms of detrimental effects of microglia activation in ALF

It has been proposed that activated microglia and related inflammatory responses are detrimental to the brain. In *in vitro* settings, microglia have been demonstrated to be able to produce several cytotoxic substances, such as ROS [359, 360], NO, proteases,

arachidonic-acid derivates, glutamate [361], quinolinic acid [361], TNF-a, IL-1b [362-366]. These factors influence the CNS cellular system in an autocrine and/or a paracrine fashion. For instance, it has been suggested that microglia-generated TNF could induce RNOS production from astrocytes [367, 368].

Results of the studies described in this thesis provide the first direct evidence for a role of microglial activation in the pathogenesis of the encephalopathy and brain edema in ALF. Cerebral microglial activation, found in ALF animals, could well explain the increased expression of the proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and elevated nitrite/nitrate levels in brains of these animals at coma (edema) stages of encephalopathy, which is consistent with previous reports of increased cerebral cytokine efflux (by measuring arterio-venous differences) in ALF patients [314, 461].

Healthy neurons express several molecules that actively down-regulate microglia and macrophage activation states [596-598]. The precise mechanisms responsible for microglial activation in ALF are unknown. However, one potential mechanism involves ammonia toxicity. ALF results in sustained hyperammonemia, and brain ammonia concentrations in the 1 to 5 mmol/L range have been reported [31]. Ammonia inhibits α-ketoglutarate dehydrogenase in rat brain mitochondria [97], resulting in decreased glucose oxidation and in lactate accumulation. Increased brain lactate production has been shown unequivocally in experimental ALF [115], and CSF lactate levels are positively correlated with the severity of HE in both

experimental [161] and human [162] ALF. It has been shown that exposure of cultured microglia to lactate in concentrations equivalent to those reported in brain in experimental ALF leads to increased production and release of TNF-α, IL-6, IL-β [457]. From our study, increased expression of the microglial marker proteins OX-6 and OX-42 (which indicates microglial activation) was found in the brains of ischemic ALF animals which correlates well with the worsening of HE and cerebral edema in these animals. Moreover, increased cerebral expression of TNF-α, IL-6, and IL-1β parallels the increase of cerebral lactate [115, 197] in ischemic ALF animals. These findings support the view that cerebral lactate accumulation activates microglia and induces subsequent cytokine generation in ALF.

In addition, activated microglia expresses various chemokines and cadhesion molecules, such as CCL2/monocyte chemotactic protein-1 (MCP-1), CCL3/Macrophage inflammatory protein-1(MIP-1), CX3CL1/ fractalkine, CXCL10/ IP-10 and IL-8/CXCL8 [599-602] which not only move resident microglia to the sites of inflammation, but also recruit blood-borne leukocytes into the neuro-inflammation sites [603-607]. In this way, activated microglia could amplify cerebral inflammatory response according to the needs against various pathogens. The significance of these peripheral-derived immune cells in the development of cerebral inflammatory response in ALF awaits further investigation.

As expected from its anti-inflammatory properties [470-472], results of the present study demonstrate that treatment with minocycline reduces microglial activation and

leads to significant decreases in expression of IL-1β, IL-6 and TNF-α in the brains of ALF rats. Moreover, these effects of minocycline were accompanied by significant decreases in brain water content, strengthening the notion that microglial activation and brain accumulation of inflammatory cytokines are implicated in the pathogenesis of brain edema in ALF. These findings are in line with previous reports that minocycline treatment is effective in limiting brain edema that accompanies intracerebral hemorrhages [473]. Moreover, brain edema resulting from experimental hypoxia/ischemia is significantly reduced in IL-1 receptor-deficient animals [474]. However, results of the present study reveal that an almost complete normalization of IL-1β mRNA and protein following minocycline treatment led to significant but incomplete inhibition of brain edema in ALF animals suggesting that other cytokines or other pathophysiologic mechanisms could also be implicated.

Future studies, using genomic and proteomic profiling [608, 609] to follow the course of microglia change over time [610-613], will help to elucidate the role of microglia in the pathogenesis of HE and cerebral edema in ALF.

4. Mechanisms of beneficial effect of mild hypothermia in the treatment of ALF

4.1 Protective effects of mild hypothermia in experimental ALF

The beneficial effects of mild hypothermia in treating ALF have been demonstrated in various animal models, such as ischemic liver failure, hepatectomy and portacaval-

shunted rat given an ammonia salt. Mild hypothermia delivers reliable therapeutic effects slowing the development of HE, brain edema and high ICP, in these ALF models [121, 197] .

4.2 Mechanisms explaining the beneficial effect of mild hypothermia in ALF

In ALF, HE, brain edema and high ICP result from multiple pathophysiologic alterations, such as altered brain osmolarity, cerebrovascular hemodynamics, cerebral energy metabolism, and neuro-inflammation [204, 378]. The capacity of mild hypothermia to influence various mechanisms during ALF may explain its beneficial effects.

4.2.1 Cerebral and arterial ammonia levels

Increased cerebral and arterial ammonia is a consistent finding in ALF, with increased brain-to-blood ammonia ratio (from 2:1 in normal to 4:1 in ALF) [31]. Although some experiments do not show decreased arterial ammonia level after hypothermia treatment [121], the results from my experiments (in this thesis [499]), clinical ALF patients [39, 311], and urea-cycle deficiency [614], clearly show that blood ammonia is decreased after hypothermia treatment. The differences may be explained by different depths of induced-hypothermia, controlled in different experimental or clinical settings. More importantly, hypothermia leads to decreased cerebral ammonia concentration in ALF. In an ammonia-intoxication experiment, the dose needed to

induce 50% mortality is two times higher in hypothermic mice than normothermic mice [424]. Decreased cerebral ammonia is observed in hypothermic mice after ammonia administration, indicating decreased ammonia transfer from blood to brain under hypothermia. In another study, decreased CSF ammonia by hypothermia treatment accompanies un-altered arterial ammonia levels, which again indicates reduced blood to brain transfer of ammonia by hypothermia [254]. The capacity of hypothermia to reduce cerebral ammonia in ALF may be due to lowering of CBF, BBB permeability, and increased arterial ammonia. In addition, hypothermia reduces bacterial ammonia generation from feces and ammonia extraction from the colon [425, 615]. Hypothermia also decreases kidney-derived ammonia release [426] and ammonia production from proteolysis [427].

4.2.2 Cerebral osmotic changes

Cerebral osmotic changes have been suspected as the cause of brain edema in ALF for many years. Glutamine is a well known osmolyte, and is also an ammonia metabolite mainly formed in astrocytes via GS [68]. Increased cerebral glutamine has been observed in various experimental models of ALF and hyperammonemia [156]. Preventing glutamine synthesis by MSO, decreases ammonia-induced astrocyte swelling in vitro [616] and brain edema in vivo [85, 86, 617]. However, hypothermia does not lower cerebral glutamine levels [115, 121], which indicates that glutamine is not the only osmotic determinant during brain edema formation in ALF. Alterations of other osmolytes have been observed, such as increased cerebral glucose, alanine and

lactate in ALF [47, 115]. It has been demonstrated that elevated cerebral lactate and alanine show a very close relationship with the progressive development of HE and brain edema in experimental ALF [31, 115]. Hypothermia is effective in correcting the disturbances of cerebral lactate and alanine [114, 124, 254], and, in this way, may attenuate the overall cerebral osmotic stress created during ALF.

4.2.3 Extracellular cerebral amino acids

Altered extracellular cerebral amino acids have been demonstrated in experimental ALF. Hypothermia treatment attenuates the increase of alanine and phenylalanine, normalizes the elevation of glutamate, aspartate, glycine, and tryptophan, but has no effects on increased glutamine, taurine, valine, tyrosine, and leucine [254]. Increased extracellular cerebral glutamate is consistently observed in ALF [113, 158, 164, 251, 252], and the mechanism involves decreased glutamate uptake by astrocytes [259, 261, 262] as well as increased astrocytic glutamate release [267]. High extracellular glutamate induces astrocyte swelling in culture [547, 618], probably through high K^+ uptake in astrocyte [428] and high RNOS production following NMDA-R stimulation [407, 497]. Hypothermia-induced reduction of extracellular cerebral glutamate may thus prevent brain edema in ALF [121, 254]. Increased excellular glycine in ALF may induce its harmful effect by acting as a positive allosteric NMDA-R modulator, and this is also attenuated by hypothermia. Increased aromatic amino acids have the capacity to enhance the synthesis of monoamine neurotransmitters in ALF [619], which is also ameliorated by hypothermia. The unaltered profile of branched chain amino acids after

hypothermia treatment excludes their role in the pathogenesis of cerebral edema in ALF.

4.2.4 Cerebrovascular autoregulation and CBF

Impairment of cerebrovascular autoregulation and increased CBF are consistently observed in ALF patients [157, 620], which is closely associated with brain edema and high ICP [379]. As a result of compromised cerebrovascular autoregulation, brain becomes vulnerable to systemic blood pressure, which generates increased CBF during high arterial pressure, and produces hypoxia during low arterial pressure. In addition, it has been shown that increased CBF correlates with the development of brain edema and high ICP in ALF [313, 621], whose mechanisms involve high cerebral blood volume load, high pressure across the BBB, and increased ammonia transfer from-blood-to-brain [379]. Hypothermia successfully re-establishes the cerebrovascular autoregulation in ALF [622, 623], and effectively prevents the increase of CBF in experimental ALF [114, 121] and in ALF patients [39, 40, 311, 314], with improvement of brain edema and high ICP.

4.2.5 Cerebral energy metabolism

An inefficient, partially blocked cerebral energy metabolic state is observed in the brain during ALF. Due to inhibited cerebral alpha-ketoglutarate dehydrogenase activity by hyperammonia, cerebral pyruvate oxidation is reduced [115], leading to increased de

novo synthesis of lactate from glucose [115] and a compensatory activation of glycolytic enzyme phosphofructokinase [168, 194]. Hence, increased cerebral and CSF lactate is consistently demonstrated in various ALF animal models [163, 197] and in ALF patients [158]. Elevated cerebral lactate concentrations show a close association with the development of HE, brain edema and high ICP in experimental ALF [115, 163].

Mild hypothermia prevents CSF lactate accumulation in ischemic ALF animals [197]. By using NMR spectroscopy and ^{13}C-glucose [114], hypothermia was shown to attenuate brain lactate synthesis from 1.7 times increase to 1.3 times increase, ^{13}C-labeled cerebral lactate from 4.5 times high to a level not different from controls. In a study involving ALF patients with uncontrolled ICP [39], hypothermia treatment decreased cerebral glucose metabolism more than ninety percent, which indicates a more efficient cerebral energy metabolism induced by hypothermia. The beneficial effects of hypothermia to improve pyruvate oxidation, decrease glucose consumption, and reduce lactate accumulation may contribute to decreased cerebral ammonia concentrations by hypothermia.

4.2.6 Inflammation

Inflammation (SIRS) and infection are frequently demonstrated in ALF. The correlation between the degree of inflammation (SIRS) and infection and the development of HE and brain edema has been described in clinical [303, 304, 624] as well as experimental

[312] studies. Hence, inflammation is recognized as a pathogenic factor in producing HE, cerebral edema, and high ICP in ALF [303, 312]. At the molecular level, increased systemic pro-inflammatory cytokines are closely associated with the increased CBF and high ICP in ALF patients [40, 306]. Positive cerebral cytokine efflux was described in ALF patients with uncontrolled ICP, but not in those ICP below 20mmHg [521]. Elevated systemic and central pro-inflammatory cytokines may increase CBF and BBB ammonia permeability, or induce astrocyte swelling directly [294, 625]. Hypothermia treatment effectively reduces systemic and brain levels of proinflammatory cytokines in ALF patients with uncontrolled high ICP, resulting in ICP restoration [311]. Hypothermia also prevents high ICP surges in ALF patients who were waiting for liver donors [314] or during liver transplant operations [40, 314], by preventing high systemic cytokine and brain cytokine production.

4.2.7 RNOS

Increased cerebral RNOS production has been observed in ALF. The causative factors include increased ammonia, pro-inflammatory cytokines, extracellular glutamate, PTBR ligands, and hyponatremia. The underlying mechanisms for high cerebral RNOS production involve induction of NOS isoforms, stimulation of NMDA-R, as well as activation of mitochondrial, antioxidant enzymes, and swollen astrocytes. Results from this thesis clearly show that hypothermia decreases systemic and central production of NO, with the reduction of cerebral iNOS and eNOS expression in ischemic liver failure animals [500]. In this ALF model,

decreased cerebral NO production by hypothermia is shown to be associated with attenuation of cerebral edema. The decreased iNOS and eNOS expression may also be a secondary effect of reduced cerebral ammonia resulting from hypothermia.

4.2.8 Seizures

Seizures are often seen in ALF patients, which is more likely to present as a preceding event before the development of cerebral edema [370]. Hypothermia reduces seizures in experimental epilepsy models [626-628]. The delay of seizures by hypothermia has also been observed in ischemic ALF rats (unpublished data).

4.2.9 Gene expression

Altered cerebral gene expression has been consistently reported in experimental ALF, which includes genes coding for AQP4, EAAT2, GFAP, GLUT-1, PTBR, SOD, HO-1 [208, 219, 227, 262, 325, 539]. Most of these genes are expressed in astrocytes, some in endothelial cells, and some are in both cell types. Mild hypothermia attenuates/restores gene expression to a normal levels in experimental ALF [220]. The molecular mechanism relating these altered gene expressions to cerebral edema in ALF awaits further investigation.

4.2.10 Systemic effects (cardiovascular and liver)

Hypothermia treatment in ALF patients has been shown to dramatically enhance cardiovascular function, resulting in increased blood pressure, elevated vascular resistance, and decreased noradrenaline need [311]. The therapeutic dilemma that using vasopressors in ALF to increase blood pressure, may also lead to high CBF and increased ICP (due to lack of cerebrovascular autoregulation in ALF [320, 629]) could be reasonably resolved by hypothermia. Regarding liver, mild hypothermia ameliorates various toxins or ischemia-reperfusion induced liver injury [446, 630-635], through mechanisms that probably involve decreased hepatic necrosis [446] and preserving hepatic regeneration [446]. The beneficial effect of hypothermia on the cardiovascular system and liver could also prevent the onset of multiple organ failure in ALF.

4.3 Undesired side-effects of mild hypothermia

Due to technical limitations, when mild hypothermia is applied to patients, the whole body has to be cooled, rather than the brain only [636]. This systemic cooling approach induces systemic side effects of hypothermia [637, 638], which potentially include respiratory infection, coagulopathy, arrhythmias, myocardial ischemia, fluid and electrolyte imbalance, hyperglycemia, hyperlactatemia, and shivering. Prolonged treatment and increased degree of hypothermia increases the possibility of developing side-effects. In ALF patients, controlling body temperature between 32C-35^{o}C has demonstrated efficacy [377, 639, 640], with no side-effects reported so far [39, 311,

314, 622]. Co-treatment with recombinant factor VIIa [641] or granulocyte colony-stimulating factor [642] may decrease the risk of coagulopathy and infection.

4.4 Indications for mild hypothermia treatment in ALF patients

Patients with ALF, who response to NAC or supportive care, who have an excellent chance of spontaneous recovery and who would not be listed for liver transplantation, would not be priority candidates for therapeutic hypothermia.

Four groups of ALF patients have been identified that will benefit from mild hypothermia therapy.

First group: ALF patients, who are waiting for OLT, and have uncontrolled ICP. Uncontrolled ICP is defined as continuing high ICP more than 25 mmHg, after treatment with two boluses of mannitol and removal of 500ml fluid by hemofiltration. Mortality rate in these patients is more than 90 percent. Mild hypothermia might be life saving for them, as it reduces uncontrolled ICP levels to a normal range, and may bridge them to OLT [39, 40, 311, 314].

Second group: ALF patients, who are waiting for OLT, have a high risk of increased ICP (although not with increased ICP yet), or are developing high ICP levels. The high risk group could be defined as ALF patients with severe/deep HE but ICP less than 20

mmHg. Mild hypothermia could be used as a prophylactic treatment in these patients [377].

Third group: patients who are undergoing OLT need to prevent increased ICP during surgery. A steep increase of ICP is frequently observed during dissection or reperfusion phases of OLT [422, 423]. Hypothermia has been shown to effectively prevent the surge of high ICP during OLT [314]. In this sense, intra-operation re-warming should be prevented.

Fourth group: patients who fulfill criteria for bad prognosis, but have no option to undergo OLT. This group of ALF patients either do not have access to OLT, or have contra-indication for OLT. Mild hypothermia might be applied to these patients for up to 7 days, until spontaneous recovery of liver function. However, re-warming without spontaneous liver recovery has a poor prognosis [39]. ALF patients with hepatitis A virus or acetaminophen-induced ALF, who have more chance of liver regeneration, may be the best candidates for hypothermia therapy in this situation.

5. Protective mechanism of minocycline in the treatment of ALF

5.1 General introduction

Minocycline is a second-generation, semi-synthetic tetracycline derivative that is a highly lipophilic drug easily crossing the BBB [643]. The cerebral minocycline level is

nearly 35% of plasma level [644]. It is traditionally used as an antibiotic against gram-positive and gram-negative bacterial infections [645, 646], through the mechanism of inhibiting protein synthesis by acting on ribosome (16S rRNA) [647]. Recently, minocycline has been described as a neuroprotective agent mainly via its anti-inflammatory action. Minocycline has demonstrated beneficial effects in treating various neurologic diseases in experimental animal models, such as intracerebral hemorrhage [648], traumatic brain injury [649], spinal cord injury [650, 651], Parkinson's disease [652, 653], amyotrophic lateral sclerosis [654], and Huntington's disease [655].

5.2 Mechanisms of minocycline's beneficial effects in ALF

5.2.1 Anti-inflammatory effects

Minocycline manifests anti-inflammatory effects with both central and systemic actions via modulating microglia, astrocytes, neutrophils, macrophages and other immune cells, which subsequently decrease the release of cytokines (TNF-α, IL-1β and IL-6), chemokines, NO, ROS, lipid mediators of inflammation, MMPs both in the brain and in the circulation [463].

Activated microglia represents the major cell-type in the brain contributing to the cerebral production of IL-1β, IL-6, TNF-α, ROS, RNS [656-661]. The inhibition of microglia activation by minocycline, which is evident by decreased CD11b/OX42 or

isolectine-B4 immuno-staining in animal models of ALF, ischemic brain injury, and 6-hydroxydopamine-injected mouse [499, 501, 662, 663], could substantially decrease the generation of these pro-inflammatory cytokines and RNOS in the brain [499, 500, 659], leading to its protective action.

Chemokines are potent chemoattractants that guide microglia, astrocytes, and peripheral neutrophils, macrophages and other immune cells to the site of increased inflammation in various neurologic diseases. Chemokines could be expressed and released from microglia, astrocytes, and brain-infiltrating immune cells. It has been demonstrated that minocycline decreases the expression of both chemokines (MIP-1, RANTES, IP-10) and chemokine receptors (CXCR3, CCR5) in microglia [664, 665] or other immune cells [666] in animal models of cerebral inflammation [665, 666], and brain injury [657, 664]. In addition, altered chemokine and chemoattractant expression has been demonstrated in the brain of ischemic ALF animals (unpublished data). These findings suggest that minocycline may exert its protective action by suppressing the expression of genes coding for chemokines or chemokine receptors, leading to decreased recruitment of resident microglia and peripheral-derived immune cells to the site of inflammation in ALF.

Lipid mediators of inflammation include a group of molecules generated through successive enzymes, including phospholipases (to produce arachidonic acid), cyclooxygenases (to give rise to prostaglandins) or lipoxygenases (to generate lipoxins and leukotrienes). PGE2, for instance, could be produced by monocytes,

macrophages/microglia, and neutrophils, which induce pyrogenicity, vasodilation, and increased vascular permeability. In ALF, increased cerebral PGE2 level has been demonstrated in ischemic liver failure animals at the coma stage (unpublished data). It has been demonstrated that minocycline inhibits phospholipase A2 (sPLA2) activity [667], decreases COX2 expression, reduces PGE2 production in cultured microglia [668]. These findings support the view that minocycline may exert protection via reducing the production of lipid mediators of inflammation in ALF.

Leukocytes could generate MMPs, which function to degrade the basement membrane of blood vessels, and facilitate leukocyte movement into the brain [669]. Increased circulating MMP-9 activity has been reported in ALF models and has been implicated in the functional alteration of BBB [670]. It has been shown that minocycline effectively decreases both MMP-9 production and MMP-9 activity, concomitant suppresses T cell migration into the brain in autoimmune encephalitis [671]. These findings indicate that minocycline may exert its anti-inflammatory effects at least partially through the inhibition of MMPs-mediated leukocyte migration into the brain in ALF.

Interestingly, minocycline exposure does not alter microglial expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell line derived neurotrophic factor (GDNF) in in vitro conditions [672], which function to promote the survival of neurons.

5.2.2 Inhibition of p38 MAPK signalling pathway

Minocycline treatment down-regulates p38 MAPK expression to ~20% of control [673]. P38 MAPKs are serine/threonine kinases, whose signalling pathway plays a role in mediating cell migration, proliferation and differentiation. P38 MAPK is activated by inflammatory cytokines, LPS, and other stress stimuli [674]. The neuroprotective effects from minocycline-induced inhibition of p38 MAPK were thought to be due to the subsequent reduction of expressions of TNF-α, IL-1β, iNOS, and COX-2 [463, 675], during which both central and peripheral inflammatory cells are involved.

Addition of minocycline to glutamate-, kainate-, or NMDA-intoxicated mixed spinal cord cultures was found to prolong the survival of neurons. This minocycline-induced beneficial effect is attributed to decreased microglia activation and the production of IL-6, IL-1β, TNF-a, and NO, in relation to the decrease of p38 MAPK activity [471, 676]. Moreover, minocycline treatment increases the survival of NO-intoxicated cultured neurons, which is directly associated with the decrease of p38 MAPK activity in these neurons [677]. These findings indicate that neuroprotective effects of minocycline relating to the down-regulation of p38 MAPK involves both microglia and neurons. Moreover, increased p38 MAPK activity is found in association with neutrophil response to inflammatory stimulation, and subsequent increased generation of IL-8 and superoxide, high neutrophil chemotaxis [678], and prolonged neutrophil survival [679]. In addition, elevated p38 MAPK activity has also been shown to relate to increased TNF-α, IL-12, IL-6, IL-1β, and NO production in macrophages [680, 681]. Thus, the

inhibition of p38 MAPK pathway by minocycline could potentially reduce the systemic inflammatory response mediated by neutrophils and macrophages.

5.2.3 Reduction of MPT

It has been proposed that mitochondrial dysfunction leads to increased RNOS production in ALF. Among the causative factors, ammonia [488, 503], glutamine [127, 128], and PTBR [565, 566] have been suggested. Following further investigation, increased opening of the MPT pore, in the inner mitochondrial membrane, has been demonstrated in cultured astrocytes following exposure to ammonia [100], glutamine [129], and PTBR ligands [567], which was considered to cause subsequent mitochondrial dysfunction and increase ROS production. It has been demonstrated that minocycline can reduce MPT directly [651, 654], which may subsequently decrease cerebral RNOS generation from mitochondria in ALF.

5.3 Side effects

In general, less than 10% of minocycline users report side effects, which present as an allergic reaction, tissue hyper-pigmentation, or autoimmune disorders [682-686]. In a retrospective study of minocycline-induced autoimmune disorders during the past 32 years, over 60 cases of minocycline-induced systemic lupus erythematosus (SLE) and 25 cases of minocycline-induced autoimmune hepatitis (AIH) were identified. In 13 cases diagnosed with both SLE and AIH, patients presented with symmetrical

polyarthritis, increased ALT/AST, and positive antinuclear antibodies (ANA). Fortunately, stopping the use of minocycline is effective in resolving their symptoms, and in dramatically improving and/or normalizing abnormal lab tests [687].

CHAPTER 4

CONCLUSIONS

Results of the findings from the articles published and included in this thesis demonstrate that:

1. Experimental ALF leads to increased brain production of proinflammatory cytokines (IL-6, IL-1β, TNF-α), and provides the first direct evidence that central inflammatory mechanisms play a role in the pathogenesis of the encephalopathy and brain edema in ALF (chapter 2.1 - article 1; chapter 2.2 - article 2).

2. Activation of microglia, measured by OX-42, OX-6, predicts the presence of severe encephalopathy (coma) and brain edema in rats with ischemic ALF, which accompanies the increased production of brain proinflammatory cytokines (chapter 2.1 - article 1; chapter 2.2 - article 2).

3. Oxidative/nitrosative stress participates in the pathogenesis of brain edema and its complications in experimental ALF animals with ischemic liver failure. Furthermore, the increases in cerebral NOS isoform expression caused by ALF are sufficient to cause increased NO production in the brain (chapter 2.3 - article 3; chapter 2.4 - article 4).

4. Anti-inflammatory treatment, such as hypothermia or antibiotics, is beneficial in the prevention of neurological complication of ALF due to hepatic devascularsation (chapter 2.1 - article 1; chapter 2.2 - article 2).

5. The beneficial effect of both hypothermia and minocycline on the neurological complications of experimental ALF is mediated, at least in part, by reduction of brain-derived oxidative/nitrosative stress (chapter 2.3 - article 3; chapter 2.4 - article 4).

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